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LARISSA YURI ISHIZU

STUDY OF LIPOLYTIC ACTIVITY OF ISOLATED ADIPOCYTES OF EPIDIDYMAL ADIPOSE TISSUE AND OF ENERGY METABOLISM OF RATS FROM TWO MODELS OF HYPERTENSION: GENETIC AND INDUCED

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RESUMO

A hipertensão é a doença cardiovascular mais comum. A resposta ao estresse é constituída pela ativação do sistema nervoso simpático e do eixo hipotálamo-pituitária-adrenal. Estudos sugerem que tais mecanismos sejam centrais na hipertensão essencial. Distúrbios metabólicos têm sido observados na hipertensão essencial, e podem ser decorrentes dos hormônios do estresse. O objetivo deste trabalho foi a caracterização dos hormônios do estresse, parâmetros metabólicos, a sensibilidade lipolítica de adipócitos epididimais isolados e a expressão de proteínas relacionadas de ratos Wistar com hipertensão induzida pela ingestão crônica de N^G-nitro-L-arginina metil éster (40 mg/kg por dia, a partir da 10ª semana de vida, por 5 semanas) (grupo L-NAME) e de ratos espontaneamente hipertensos (grupo SHR), comparados com seus controles, Wistar (grupo W) e Wistar Kyoto (grupo WKY), respectivamente. Comparações entre ambos os controles e ambos os hipertensos também foram realizadas. O peso corpóreo, ingestão alimentar e hídrica e taxa metabólica de repouso foram feitas na 7ª e na 14ª semana de vida. A coleta de sangue para análises séricas e de tecidos (para medida de peso, morfometria de adipócitos, ensaio funcional com adipócitos epididimais isolados e análise de expressão por Western Blot) e a eutanásia ocorreu nos ratos anestesiados, submetidos a jejum prévio de 12-16 h, na 15ª semana de vida, por volta das 9:00h da manhã. Altas concentrações circulantes de catecolaminas e corticosterona foram observadas nos grupos L-NAME, SHR e WKY, mas não em W. O L-NAME exibiu respostas metabólicas típicas do estresse agudo, pois além da menor adiposidade, reduziu a sua ingestão alimentar. O WKY exibiu algumas alterações típicas do estresse crônico, como menor taxa metabólica e reduzida expressão de UCP3 na musculatura esquelética. O SHR apresentou alterações que podem ser relacionadas a uma possível hiperfunção da tireoide, como aumento da ingesta alimentar e da taxa metabólica de repouso e menor adiposidade. Houve aumento da lipólise mediada por adrenoceptores β_2 no grupo L-NAME se comparado ao Wistar, mas sua expressão não foi diferente. Entretanto, apresentou menor expressão de adrenoceptores β_1, receptor de adenosina A2 e perilipina e aumento de HSL. Por outro lado, o grupo SHR apresentou inibição da lipólise mediada por adrenoceptores β_2 comparado ao WKY, devido ao seu efeito promíscuo de associação a proteínas Gi, sem alteração na expressão destas proteínas. No entanto, apresentou alta expressão de ATGL e de perilipina. Em resumo, nossos resultados constataram algumas alterações metabólicas em ambos os grupos hipertensos e no normotenso WKY, que podem ser atribuídas aos hormônios do estresse, cuja presença em altas concentrações no grupo WKY não foi suficiente para desenvolver a sua hipertensão. O

presente trabalho também evidenciou uma função alterada do adrenoceptor β_2 em adipócitos epididimais isolados no estado hipertensivo, o que já foi descrito em cardiomiócitos e que pode ter função protetora.

ABSTRACT

Hypertension is the most common cardiovascular disease. Stress response is composed by activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. Studies suggest that these mechanisms are central in essential hypertension too. Metabolic disorders have been observed in essential hypertension, and may be due to stress hormones. The aim of this study was the characterization of stress hormones, some metabolic parameters, the lipolytic sensitivity of isolated epididymal adipocytes and the expression of related proteins of Wistar rats with hypertension induced by chronic ingestion of N^G-nitro-L-arginine ester methyl (40 mg / kg per day, in 10-week-old rats, for 5 weeks) (L-NAME group) and spontaneously hypertensive rats (SHR), compared to their controls, Wistar (W group) and Wistar Kyoto (WKY group), respectively. We also performed comparisons between W and WKY and between L-NAME and SHR. Body weight, food and water intake and resting metabolic rate were measured in 7 and 14-week-old rats. Blood sampling for serum analysis and tissue extraction for analysis of weight, adipocyte size, lipolytic response of isolated epididymal adipocytes and protein expression by Western Blot, and euthanasia occurred at around 9: 00 a.m. in anesthetized 15-week-old rats that were previously fasted for 12-16 h. High levels of catecholamines and corticosterone were observed in L-NAME, SHR and WKY groups, but not in W. L-NAME showed typical metabolic responses of acute stress, like reduced adiposity and food intake. WKY exhibited some alterations of chronic stress, such as reduced metabolic rate and UCP3 expression in skeletal muscle. SHR presented changes that were attributed to a possible thyroid hyperfunction, such as increased food intake and resting metabolic rate and reduced adiposity. L-NAME showed increased β_2-adrenoceptor mediated lipolysis, although there were no differences in the expression of related proteins. However, it had lower expression of β_1-adrenoceptor, adenosine A2 receptor and perilipin and increased HSL. On the other hand, SHR group displayed promiscuous β_2-adrenoceptor association to Gi without changes in the expression of the associated proteins. Nevertheless, it showed high expression of ATGL and perilipin. In summary, our results observed some metabolic abnormalities in both hypertensive groups and in the normotensive WKY, which may be attributed to stress hormones, whose presence at high circulating levels in WKY group was not sufficient to develop its hypertension. This study also showed an altered function of β_2-adrenoceptors in epididymal isolated adipocytes in the hypertensive groups, which has already been described in cardiomyocytes and that may have a protective role.

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Introdução

A hipertensão

As doenças cardiovasculares são as maiores causadoras de morte no mundo: estima-se que sejam responsáveis anualmente por 17 milhões de mortes no mundo, o que corresponde à 31% de todas as mortes (Oms, 2013). Dentre elas destacam-se a doença cardíaca isquêmica e a hipertensão que foram, respectivamente, a primeira e a décima causadora de mortes no mundo no ano de 2012 (Oms, 2014). A hipertensão é a doença cardiovascular mais comum, a qual afeta cerca de 1 bilhão de pessoas no mundo todo (Oms, 2013). Estima-se que ela seja responsável por 7,1 milhões de mortes (13%) de todas as mortes, anualmente (Thomas e Dasgupta, 2015).

Hipertensão é definida como elevação da pressão sanguínea, cujos valores são: maiores ou iguais a 140 mm Hg para a pressão arterial sistólica e/ou maiores ou iguais a 90 mm Hg para a pressão arterial diastólica (Oms, 2013). Hipertensão primária ou essencial é a elevação da pressão sanguínea sem causas secundárias, e corresponde a 95% de todos os casos de hipertensão (Wang *et al.*, 2015).

Os determinantes da pressão arterial são o débito cardíaco e a resistência periférica, e qualquer variação em um ou em ambos resulta em alterações nos valores pressóricos normais (Folkow, 1982). Os mecanismos de controle da pressão arterial atuam na regulação do calibre e reatividade vascular, a distribuição de fluido dentro e fora dos vasos e o débito cardíaco. Estes mecanismos pressores e depressores interagem e se equilibram (Folkow, 1982). Quando este equilíbrio é interrompido, com predominância dos fatores pressores, inicia-se a hipertensão primária, sendo, portanto, considerada uma doença de origem multifatorial. Essa ruptura pode ser provocada e/ou acelerada pelos fatores ambientais, como excesso de sal na dieta e estímulos psicoemocionais, entre outros (Folkow, 1982).

O fator mais importante na gênese da hipertensão arterial é o aumento da resistência periférica, e portanto, os mecanismos de redução do calibre vascular merecem a devida atenção. Tais mecanismos incluem a contração da musculatura lisa do vaso, ou o aumento da espessura desta musculatura, o qual por sua vez, ocorre por hipertrofia muscular ou por remodelamento (isto é: redução dos diâmetros interno e externo, sem modificação da massa) (Krieger, Irigoyen e Krieger, 1999). O tônus vascular é determinado pela atividade simpática e por substâncias vasopressoras ou vasodepressoras circulantes ou sintetizadas pelas células da musculatura lisa ou endoteliais (Krieger, Irigoyen e Krieger, 1999).

A hipertensão pode prejudicar a função cardíaca, pois quanto maior a pressão nos vasos sanguíneos, maior o trabalho do coração para bombear o sangue. Desta forma, se não controlada, a hipertensão pode resultar em hipertrofia do coração, em ataque cardíaco (que acontece se o aporte sanguíneo para o coração é bloqueado e as células musculares cardíacas morrem por escassez de oxigênio), e insuficiência cardíaca (quando o coração não é capaz de bombear sangue e oxigênio suficientes para outros órgãos) (Oms, 2013). Não somente o coração, como os vasos sanguíneos podem ser prejudicados, com o desenvolvimento de aneurismas, e ruptura de vasos sanguíneos, o que pode resultar, por exemplo, no acidente vascular cerebral. Além disso, a hipertensão pode levar à insuficiência renal, cegueira e comprometimento cognitivo (Oms, 2013).

Modelos de estudo da hipertensão arterial

Na década de 40, o Dr. Irv Page propôs a famosa "teoria do mosaico", a qual afirma que a hipertensão arterial primária é uma doença multifatorial, ou seja, ela não apresenta uma única causa, mas múltiplos fatores que se interligam, que resulta numa desorganização do complexo e delicado sistema de controle da pressão arterial (H. e Page, 1949). Atualmente, esta idéia é bastante difundida, cuja adaptação para os dias atuais incluem os fatores genético, ambiental, anatômico, neural, endócrino, humoral e hemodinâmico. Portanto, o entendimento dos mecanismos fisiopatológicos desta condição requer a utilização de um ou mais modelos experimentais de hipertensão (Cabral, Vasquez e Mauad, 1997; Dornas e Silva, 2011). A escolha do modelo ideal de estudo depende da alteração cardiovascular e/ou fator etiológico na hipertensão que se pretende estudar, ou dar maior enfoque (Dornas e Silva, 2011). Neste sentido, podemos citar uma ampla gama de modelos bastante utilizados pela comunidade científica. Existem, por exemplo, os modelos de hipertensão renal: a hipertensão renovascular (Goldblatt *et al.*, 1934) e a renopriva, as quais enfatizam o prejuízo da função renal como desencadeador do processo hipertensivo, seja por ativação do sistema renina-angiotensina-aldosterona (SRAA), ou por retenção de sódio e água e consequente aumento de volemia (Dornas e Silva, 2011). Há também os modelos de hipertensão induzidos por agentes estressores, como estresse psicossocial, imobilização, privação de comida, estimulação elétrica e frio e os modelos induzidos por ingestão crônica de dieta rica em sal, gordura ou açúcar: ambos os tipos de modelos são adequados aos estudos sobre participação destes fatores (estresse e dieta) na etiologia da hipertensão (Dornas e Silva, 2011). Há ainda os modelos transgênicos, que permitem o estudo de um gene relacionado à hipertensão, por meio de sua superexpressão e também os modelos de hipertensão endócrina, os quais, por sua vez,

são apropriados para estudos sobre a participação de determinados hormônios na etiologia da hipertensão, como a angiotensina II e a aldosterona (Dornas e Silva, 2011).

Outro modelo de hipertensão estudado extensivamente é por inibição crônica da óxido nítrico (NO) sintase (enzima responsável pela síntese de NO), por meio da administração oral crônica de N^G-nitro-L-arginina metil éster (L-NAME), inibidor da enzima, descrito primeiramente, de modo independente, por Ribeiro et al (1992) (Ribeiro *et al.*, 1992) e Baylis et al (1992) (Baylis, Mitruka e Deng, 1992). O óxido nítrico exerce uma função importante na regulação da resistência vascular sistêmica, causando vasodilatação tônica. Essa hipertensão é associada a uma intensa vasoconstrição periférica, e consequentemente, ao aumento da resistência vascular periférica (Ribeiro *et al.*, 1992). Há evidências de que também ocorre redução do débito cardíaco paralelamente à inibição crônica da NO sintase (Biwer *et al.*, 2013) e taquicardia, dependente de hiperatividade simpática (Palma *et al.*, 2015). Aliás, esta hiperatividade simpática, de origem central, tem sido sugerida como um mecanismo adicional de iniciação e manutenção da hipertensão (Bergamaschi, Campos e Lopes, 1999; Thomas e Dasgupta, 2015), juntamente com o SRAA (Suehiro *et al.*, 2015). Este modelo tem sido muito utilizado para se entender a a hipertensão primária em humanos pois a deficiência de NO é uma das causas desta condição (Berkban *et al.*, 2015).

O modelo de hipertensão genética, representado pelos ratos espontaneamente hipertensos (SHR) e seu controle Wistar Kyoto (WKY) também têm sido extensivamente utilizado devido às suas similaridades com a hipertensão essencial em seres humanos (Trippodo e Frohlich, 1981; Dornas e Silva, 2011). Existe uma gama de alterações que contribuem para o estabelecimento e manutenção do quadro hipertensivo deste modelo, o qual desenvolve esta condição dentre 4 a 6 semanas de vida (Zicha e Kunes, 1999). Primeiramente há um aumento da resistência vascular periférica, que é sucedido por hipertrofia dos vasos, que tornam-se mais responsivos a agentes vasoconstritores (Yamori *et al.*, 1981). O SHR também apresenta aumento do tônus simpático causado pelo estresse oxidativo na medula ventrolateral rostral, importante fonte de entrada excitatória para nervos pré-ganglionares simpáticos, a qual está grandemente envolvida no controle da pressão arterial (Dickinson, 2007; Kishi e Hirooka, 2013). Este modelo também apresenta o SRAA ativado, o que também contribui com seu estado hipertensivo (Kishi e Hirooka, 2013). Além disso, já foi observado em vários tecidos, como eritrócitos, coração, fígado e cérebro, uma alteração nos mecanismos de trocas iônicas que favorece a permeabilidade ao sódio, que resulta no aumento da excitabilidade das células (Wiss *et al.*, 1989).

Os ratos Wistar, WKY e SHR constituem, portanto, 3 linhagens que são aparentadas, uma vez que tanto o SHR, como seu controle WKY, são provenientes de endocruzamentos de ratos da linhagem Wistar. Os ratos SHR foram estabelecidos como uma linhagem isogênica em 1969, no NIH (National Institutes of Health, Estados Unidos), a partir de endocruzamentos de ratos Wistar hipertensos, os quais se iniciaram em 1959, pelos pesquisadores Okamoto e Aoki, na Universidade de Kyoto (Kurtz e Morris, 1987; Louis e Howes, 1990). Já o seu controle WKY foi obtido em 1971, a partir de endocruzamentos dos ratos normotensos descendentes daqueles provenientes da colônia de Kyoto, a qual dera origem ao SHR (Kurtz e Morris, 1987; Louis e Howes, 1990). Desta forma, este sistema de endocruzamentos selecionou genes responsáveis pela hipertensão na linhagem SHR, mas não no WKY (Louis e Howes, 1990), o qual, embora seja o seu controle normotenso, apresenta anormalidade hormonais e comportamentais, que o caracterizam como modelo para estudos de depressão e hiperreatividade ao estresse (Will, Aird e Redei, 2003). Esta linhagem apresenta maiores concentrações circulantes de corticosterona e ACTH que a linhagem Wistar (Will, Aird e Redei, 2003). Desta forma o modelo representado pelo controle WKY e o hipertenso SHR é adequado para estudos de alterações atribuídas ao estado hipertensivo, ao passo que o mesmo WKY pode ser comparado ao Wistar em estudos sobre ambiente hormonal clássico presente na reação ao estresse crônico desatrelado da hipertensão.

O tecido adiposo

O tecido adiposo é constituído de uma matriz de tecido conjuntivo, tecido nervoso, células do estroma vascular, nódulos linfáticos, células do sistema imune (linfócitos e macrófagos), fibroblastos, pré-adipócitos e adipócitos, que são as suas unidades funcionais (Ahima, 2006). A gotícula de gordura dos adipócitos é revestida por fosfoproteínas, que são as perilipinas, as quais modulam os processos de estocagem e mobilização de triacilgliceróis. Elas previnem a lipólise em condições basais, e auxiliam a lipólise estimulada (Sztalryd e Kimmel, 2014).

Existem 3 tipos de tecido adiposo: o marrom, o bege e o branco, com diferenças estruturais, funcionais e moleculares (Peirce, Carobbio e Vidal-Puig, 2014).

O tecido adiposo marrom possui adipócitos com morfologia multilocular, ou seja, possuem numerosas e pequenas gotículas lipídicas no seu citoplasma. Suas mitocôndrias são grandes, esféricas e numerosas e é mais vascularizado que o tecido adiposo branco: estas duas características conferem a sua cor marrom (Wronska e Kmiec, 2012). Possui função de produção de calor, ou termogênese, pela ação da proteína desacopladora 1 (UCP1), que

localiza-se na membrana interna de suas mitocôndrias, e desacopla a fosforilação oxidativa da cadeia respiratória, dissipando esta energia na forma de calor (Harms e Seale, 2013). A termogênese pela gordura marrom é particularmente importante para pequenos mamíferos e recém-nascidos (Saely, Geiger e Drexel, 2012), enquanto a gerada pela musculatura esquelética, via proteína desacopladora 3 (UCP3), para os humanos adultos (Depieri *et al.*, 2004), o que aumenta o gasto energético e a taxa metabólica do organismo (Depieri *et al.*, 2004).

O tecido adiposo bege possui muitas semelhanças com o marrom: também apresenta morfologia multilocular e numerosas mitocôndrias no seu citoplasma, as quais também expressam UCP1 que desencadeia a termogênese (Harms e Seale, 2013). Porém possuem algumas diferenças, que o categoriza como um terceiro tipo de gordura: expressa genes específicos, somente expressam UCP1 e outros genes termogênicos sob estímulos, como o frio ou estimulação do receptor adrenérgico β_3 e apresenta precursor embionário diferente do marrom (Harms e Seale, 2013). Interessantemente, o tecido bege possui o mesmo precursor embrionário que o branco, ou então é proveniente do adipócito branco maduro, pelo processo de transdiferenciação (Harms e Seale, 2013; Peirce, Carobbio e Vidal-Puig, 2014). Os tecidos adiposos marrom e bege estão presentes em pouca quantidade no ser humano adulto, e a sua participação no metabolismo energético ainda é pouco esclarecida; entretanto eles têm sido alvo de estudos pois emergem como alvos terapêuticos em potencial no que dizem respeito à obesidade e outras desordens metabólicas (Harms e Seale, 2013; Peirce, Carobbio e Vidal-Puig, 2014).

O tecido adiposo branco possui morfologia unilocular, ou seja, seus adipócitos apresentam uma única e grande gotícula lipídica no seu citoplasma, a qual é responsável por 90% do seu volume. Apresentam mitocôndrias finas e alongadas, que variam em número (Saely, Geiger e Drexel, 2012). Sua função principal é atuar como um centro regulatório chave no metabolismo energético e como o principal reservatório de energia do organismo, na forma de triacilgliceróis (Wronska e Kmiec, 2012; Cao, 2014). Desempenha sua função regulatória por meio da secreção de adipocinas, as quais também influenciam outros processos, como angiogênese, controle da pressão arterial, coagulação e imunidade (Wronska e Kmiec, 2012). Sua função de reservatório energético se dá por meio de dois processos: quando há abundância de energia originada por aumento da ingestão e diminuição do gasto energético, o tecido adiposo estoca triacilgliceróis pela lipogênese; em momentos de escassez energética ou aumento da demanda de energia, há mobilização destes estoques, pela lipólise (Wronska e Kmiec, 2012). Possui ainda diversas outras funções no organismo, como

isolamento térmico, amortecimento contra choques mecânicos e sustentação de órgãos (Wronska e Kmiec, 2012).

A lipólise

A lipólise é o processo catabólico, no qual há quebra do triacilglicerol em glicerol e ácidos graxos livres (ou ácidos graxos não-esterificados) (Frühbeck *et al.*, 2014). Ela pode acontecer em condições basais ou estimulada por diversos fatores (Frühbeck *et al.*, 2014; Sztalryd e Kimmel, 2014).

Na condição basal, a lipase de triacilglicerol (ATGL) e a lipase sensível a hormônio (HSL) são citosólicas; as perilipinas encontram-se não-fosforiladas e associadas com a proteína designada gene comparativo de identificação-58 (CGI-58) (Sztalryd e Kimmel, 2014) (Fig. 1). A associação entre a ATGL e a CGI-58, co-ativadora da ATGL, que pode ocorrer na condição basal, é responsável pela lipólise basal (Bézaire e Langin, 2009).

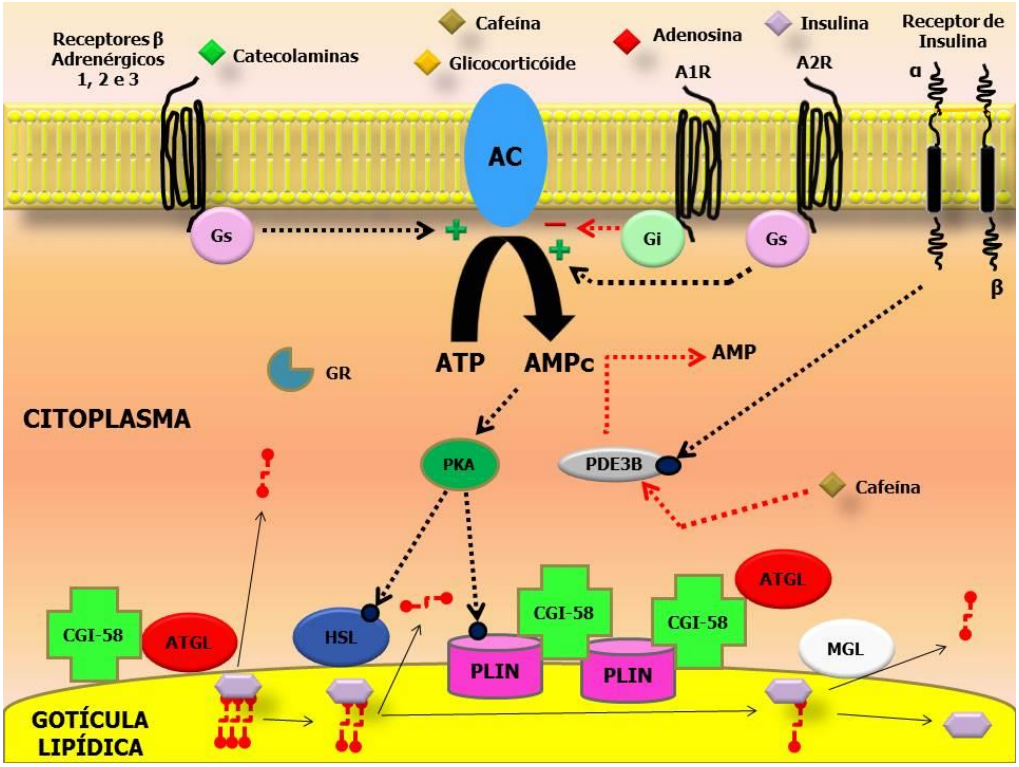

Figura 1: Representação esquemática de um adipócito branco, com as principais moléculas e vias relacionadas à lipólise basal e estimulada por catecolaminas, ambas moduladas pelos receptores de adenosina. AC: adenilato ciclase; Gs: proteína G estimulatória; Gi: proteína G inibitória; PKA: proteína quinase A; PLIN: perilipina; CGI-58: proteína do gene comparativo de identificação-58; ATGL: lipase de triacilglicerol; HSL: lipase hormônio sensível; MGL: lipase de monoacilglicerol; PDE3b: fosfodiesterase 3b; GR: receptor de glicocorticoide; A1R: receptor de adenosina A1; A2R: receptor de adenosina A2. Setas pontilhadas pretas indicam ativação; setas pontilhadas vermelhas indicam inibição.

A principal via da lipólise estimulada é a via da proteína quinase dependente de AMPc (PKA), na qual a ativação dos receptores acoplados à proteína G estimulatória (Gs) ativam a adenilato ciclase, que convertem AMP em AMPc, o qual ativa a PKA (Chaves, Frasson e Kawashita, 2011). Esta enzima, por sua vez, fosforila a perilipina e a HSL. A perilipina fosforilada libera a CGI-58, que então ativa a ATGL (Sztalryd e Kimmel, 2014), a qual atua predominantemente na molécula de triacilglicerol, na sua hidrólise em ácido graxo e diacilglicerol (Yang *et al.*, 2013). A perilipina fosforilada também associa-se com a HSL fosforilada, o que permite o seu acesso à gotícula lipídica, atuando principalmente em diacilglicerol (Sztalryd e Kimmel, 2014). Por fim a lipase de monoacilglicerol atua na hidrólise do monoacilglicerol (Yang *et al.*, 2013) (Fig. 1). Os mecanismos exatos que envolvem as perilipinas, a CGI-58 e a ATGL, na lipólise basal e estimulada ainda não estão muito bem esclarecidos (Frühbeck *et al.*, 2014).

As catecolaminas são os principais agentes lipolíticos endógenos, e atuam pela via da PKA, por meio dos receptores adrenérgicos (ou adrenoceptores) β_1, β_2 e β_3 (Fig. 1). Apesar de atuarem por meio do mesmo sistema de segundo mensageiro, diferem quanto à sua afinidade relativa a catecolaminas, sua susceptibilidade à dessensibilização, ao seu acoplamento a proteínas G, e à sua função e expressão em função da espécie, sexo, idade, localização anatômica e estados fisiológicos e patológicos (Lafontan, 2012). Sob condições fisiológicas normais, a lipólise induzida por catecolaminas ocorre principalmente via adrenoceptores β_1 e β_2 no tecido adiposo branco de humanos e via β_1 e β_3 no mesmo tecido em ratos (Lafontan, 2012). A função do adrenoceptor β_3 na tecido adiposo branco de humanos ainda não está totalmente esclarecida, enquanto o adrenoceptor β_2 representa uma pequena quantidade de receptores β-adrenérgicos em adipócitos de ratos. Eles são regulados por catecolaminas e glicocorticoides, que são capazes de causar a dessensibilização e sensibilização de cada subpopulação de β-adrenoceptores (Lafontan, 2012).

A lipólise adrenérgica pode ser modulada por diferentes fatores, dentre eles, a adenosina. A adenosina é produto da metabolização do ATP, e é liberada por todas as células do corpo, especialmente sob certas condições, como exercício físico, estresse ou por dano celular. Ela age por meio de seus receptores, que são acoplados à proteína G estimulatória (Gs) e inibitória (Gi) (Koupenova e Ravid, 2013). Nos adipócitos, ambos os tipos de receptores estão presentes, sendo que o receptor acoplado à proteína Gi (receptor de adenosina A1) predomina sobre o receptor acoplado à proteína Gs (receptor de adenosina A2)

(Panchal *et al.*, 2012; Frühbeck *et al.*, 2014). Então o efeito da adenosina é sobre a inibição da atividade da adenilato ciclase, via proteína Gi (Koupenova e Ravid, 2013) (Fig. 1).

Alguns compostos da dieta têm a capacidade de influenciar diretamente a regulação da lipólise (Frühbeck *et al.*, 2014). Dentre eles está a cafeína e outras metilxantinas que elevam a concentração de AMPc intracelular por diferentes mecanismos. Ela antagoniza o principal o receptor de adenosina A1, o que libera a sua inibição sobre a adenilato ciclase, o que é suficiente para estimular a lipólise mesmo na ausência de ligantes estimulatórios (Honnor, Dhillon e Londos, 1985). A cafeína também antagoniza os receptores de adenosina A2, porém este efeito é irrelevante devido à sua baixa expressão dos adipócitos. O outro mecanismo da cafeína é a inibição da enzima fosfodiesterase (PDE), que é estimulada por insulina e degrada o AMPc; então a cafeína aumenta a disponibilidade de AMPc, que é produzido na lipólise estimulada (Panchal *et al.*, 2012; Frühbeck *et al.*, 2014) (Fig 1).

Estresse, hipertensão e alterações metabólicas

O estresse é definido como uma resposta do organismo frente a um agente estressor, que é uma demanda real ou percebida, avaliada como uma ameaça, que tem como finalidade gerar adaptação. Ele gera respostas de enfrentamento, no âmbito biológico, comportamental e social que trazem o organismo para uma nova alostasia (Mcewen e Gianaros, 2011). Quando esta carga alostática supera a capacidade adaptativa do indivíduo, esta sobrecarga alostática predispõe ao desenvolvimento de doenças (Mcewen e Morrison, 2013).

A resposta ao estresse agudo e crônico apresenta dois componentes: o eixo hipotálamo-pituitária-adrenal (HPA) e o sistema nervoso simpático (SNS) (Mcewen e Gianaros, 2011). Para ativação do eixo HPA, o estímulo estressor é transmitido via tronco cerebral até o córtex, e posteriormente ao hipotálamo, o qual libera o hormônio liberador de corticotropina (CRH). Este desencadeia liberação do hormônio adrenocorticotrópico (ACTH) pela pituitária, o qual estimula secreção de hormônios pelo córtex da adrenal, que são os mineralocorticoides (aldosterona), os glicocorticoides (cortisol e corticosterona) e os androgênios e também pela medula da adrenal, que são as catecolaminas (adrenalina e noradrenalina). Esta liberação de catecolaminas, somada ao aumento da liberação de noradrenalina pelos terminais nervosos simpáticos constituem a ativação do SNS (também designada “simpatoexcitação”) (Charmandari, Tsigos e Chrousos, 2005). Portanto, podem ser considerados hormônios do estresse: o ACTH, os glicocorticoides, a adrenalina e a noradrenalina (Axelrod e Reisine, 1984).

A ativação aguda destes sistemas, com a predominância da ativação simpática, é altamente adaptativa, com o objetivo de aumentar a disponibilidade de nutrientes e aporte sanguíneo aos órgãos-alvo (Tsigos e Chrousos, 2002). Contudo, a sua ativação crônica, com predominância do eixo HPA e consequente exposição prolongada aos glicocorticoides pode ter efeitos deletérios ao organismo (Dallman *et al.*, 2004). Desta maneira, o estresse crônico tem sido associado com distúrbios endócrinos, neurais, imunes, no sistema cardiovascular e no metabolismo (Chandola, Brunner e Marmot, 2006; Matsuura *et al.*, 2015).

A hipertensão é uma alteração cardiovascular, que pode ser decorrente do estresse crônico (Oms, 2013). Desta forma, as alterações cardiovasculares que levam à hipertensão são atribuídas à ativação do eixo HPA (Gold *et al.*, 2005; Goodwin e Geller, 2012) e principalmente do SNS (Björntorp *et al.*, 2000; Hering e Schlaich, 2015; Thomas e Dasgupta, 2015). O excesso de glicocorticoides desencadeado pela ativação crônica do eixo HPA tem sido associado ao desenvolvimento da hipertensão em alguns estudos, na sua maioria *in vitro*, os quais sugerem o aumento da reabsorção de sódio e da resistência vascular periférica (Goodwin e Geller, 2012). A ativação crônica do SNS, por sua vez, pode aumentar a reatividade vascular, a hipertrofia das células musculares lisas dos vasos e a resistência vascular periférica, levando à hipertensão (Thomas e Dasgupta, 2015).

As alterações metabólicas decorrentes do estresse crônico também são atribuídas à ativação crônica do SNS e do eixo HPA. A ativação do SNS promove respostas catabólicas agudas, como lipólise pelo tecido adiposo branco, glicogenólise e gliconeogênese pelo fígado, secreção de glucagon pelo pâncreas ao passo que aumenta captação de glicose pelas células musculares esqueléticas de modo insulino-independente (Thorp e Schlaich, 2015); entretanto, a ativação crônica do SNS pode levar a anormalidades no metabolismo.

Julius et al. (Julius, Valentini e Palatini, 2000) foram os primeiros a sugerirem que a obesidade, pode ser assim desencadeada, devido a uma possível dessensibilização de β-adrenoceptores, causada pela simpatoexcitação crônica, o que deve diminuir a termogênese (Julius, Valentini e Palatini, 2000; Thorp e Schlaich, 2015).

A resistência à insulina é outro distúrbio metabólico que pode estar associado à ativação crônica do SNS, atribuída à vasoconstrição na musculatura esquelética via receptores α-adrenérgicos, o que deve dificultar a captação de glicose na situação pós-prandial, a qual estimula a secreção adicional de insulina pelo pâncreas, levando à hiperglicemia (Julius e Valentini, 1998; Thorp e Schlaich, 2015).

Os glicocorticoides, por sua vez, sinergizam com as catecolaminas durante a resposta ao estresse para promover uma resposta lipolítica, gliconeogênica e glicogenolítica, além de

proteólise da musculatura esquelética, o que deve assegurar aporte energético suficiente para as demandas requeridas durante a reação ao estresse (Sacta, Chinenov e Rogatsky, 2015). Contudo, do mesmo modo que a simpatoexcitação, a exposição crônica aos glicocorticoides também pode resultar em distúrbios metabólicos.

Desta forma, altas concentrações circulantes de glicocorticoides, podem, a longo prazo, causar o aumento de peso e a obesidade central (Fardet e Fève, 2014; Lee *et al.*, 2014). Apesar do efeito lipolítico dos glicocorticoides no estresse agudo, no estado crônico, paradoxalmente, são observadas a lipogênese e a adipogênese, além da lipólise; os mecanismos contraditórios envolvidos nestes processos são complexos e pouco compreendidos (Fardet e Fève, 2014; Lee *et al.*, 2014). Nesta situação, a lipólise parece predominar no tecido adiposo subcutâneo, enquanto que a lipogênese e a adipogênese parecem ser dominantes no tecido adiposo visceral (Fardet e Fève, 2014). Outros mecanismos também estão envolvidos com o desenvolvimento da obesidade pelos glicocorticoides, como a diminuição da expressão de UCP1 pelo tecido adiposo marrom e o aumento da ingestão, especialmente de alimentos mais calóricos e gordurosos a qual constitui resposta adaptativa para estocagem de energia frente a condições estressantes (Fardet e Fève, 2014).

A resistência à insulina também pode ser desencadeada pela exposição crônica aos glicocorticoides, que podem prejudicar diretamente a sinalização da insulina no músculo esquelético, tecido adiposo e fígado e indiretamente, por diversos mecanismos, dentre os quais podemos citar: a diminuição da secreção de adiponectina pelo tecido adiposo branco, a qual melhora a sensibilidade à insulina nos seus tecidos-alvo; o aumento da lipólise, a deposição de excesso de ácidos graxos livres circulantes no fígado e disfunção das células β-pancreáticas (Ferris e Kahn, 2012).

A dislipidemia é outra condição que pode ser desencadeada pelo excesso de glicocorticoides cronicamente, e que envolve diversos mecanismos ainda pouco compreendidos, dentre os quais podemos destacar: o aumento da lipólise pelo tecido adiposo (principalmente o visceral) e da sua sensibilidade a agentes lipolíticos, como catecolaminas e o hormônio do crescimento (GH) e também aumento da síntese de lipoproteínas de muito baixa densidade (VLDL) pelo fígado, acúmulo de ácidos graxos livres neste órgão, e diminuição da β-oxidação de ácidos graxos (Fardet e Fève, 2014).

Neste contexto, o Laboratório de Estudo do Estresse (LABEEST) tem investigado as alterações metabólicas causadas por estresse agudo em modelos de ratos, em especial, a resposta lipolítica de adipócitos isolados induzida por catecolaminas e outros agonistas adrenérgicos (Farias-Silva *et al.*, 1999; Farias-Silva *et al.*, 2002; Sampaio-Barros *et al.*, 2003;

Farias-Silva *et al.*, 2004). Estas alterações no estresse agudo também são desencadeadas pela ativação do SNS e do HPA, cuja liberação de catecolaminas e de glicocorticoides, respectivamente, desencadeia a dessensibilização e sensibilização das diferentes subpopulações de receptores adrenérgicos (Lafontan, 2012). Nosso grupo observou aumento da lipólise basal e da resposta lipolítica mediada por adrenoceptores β_2 e redução da lipólise mediada por adrenoceptores β_1 em adipócitos isolados da região epididimal de ratos estressados por choque nas patas (Farias-Silva *et al.*, 1999) e por natação (Sampaio-Barros *et al.*, 2003; Farias-Silva *et al.*, 2004).

Hipertensão e metabolismo

Interessante ressaltar que a simpatoexcitação e a ativação do eixo HPA parecem ser mecanismos centrais da hipertensão essencial, independentemente do fator etiológico estar ou não relacionado a um agente estressor externo bem caracterizado (Björntorp *et al.*, 2000; Gold *et al.*, 2005; Hering e Schlaich, 2015). Isso pode ser atribuído a alterações no balanço autonômico neural já desencadeadas por pequenas, mas prolongadas elevações na pressão sanguínea, o que resulta na simpatoexcitação (Hering e Schlaich, 2015), que deve ativar o eixo HPA devido à íntima interconexão de ambos (Björntorp *et al.*, 2000).

É extremamente recorrente na literatura a presença de estudos que relatam o surgimento da hipertensão devido a alterações metabólicas (Dorresteijn, Visseren e Spiering, 2012; Kalil e Haynes, 2012; Hall *et al.*, 2015). Entretanto os estudos sobre a relação inversa, ou seja, dos distúrbios metabólicos que surgem na hipertensão essencial são escassos; porém eles identificam, de maneira geral, os mesmos distúrbios metabólicos desencadeados pelo estresse crônico, descritos acima, os quais passamos a apresentar.

Primeiramente, alguns estudos em humanos hipertensos sugerem que a hipertensão predispõe à obesidade (Kannel *et al.*, 1967; Wassertheil-Smoller *et al.*, 1992; Lasser *et al.*, 1995; Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011; Rekleiti, 2014). De acordo com estes estudos, pacientes hipertensos têm dificuldade em perder peso (Wassertheil-Smoller *et al.*, 1992; Lasser *et al.*, 1995; Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011); e diminuem sua pressão sanguínea com a perda de peso (Lasser *et al.*, 1995). Eles também têm risco maior de desenvolver a obesidade que os indivíduos normotensos, de acordo com o consagrado estudo de Framingham (Kannel *et al.*, 1967). Sugere-se que esta predisposição seja atribuída à diminuição da termogênese do tecido adiposo marrom e do músculo esquelético causado pela

simpatoexcitação crônica (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011), a qual fora mencionada anteriormente.

Outros estudos com humanos e modelos animais relatam o metabolismo da glicose prejudicado na hipertensão. Alguns trabalhos reportam menor captação de glicose estimulada por insulina em ratos hipertensos SHR (Hulman, Falkner e Chen, 1991; Reaven e Chang, 1991; Hajri *et al.*, 2001; Potenza *et al.*, 2005; Potenza *et al.*, 2006) e aumento (Reaven e Chang, 1991; Potenza *et al.*, 2005; Potenza *et al.*, 2006) ou manutenção (Hulman, Falkner e Chen, 1991) das concentrações plasmáticas de insulina, quando comparados com o seu controle WKY, embora, em alguns estudos não apresentem diferenças na glicemia de jejum de ambos os grupos (Hulman, Falkner e Chen, 1991; Potenza *et al.*, 2005; Potenza *et al.*, 2006). Ratos com hipertensão induzida por inibidores da NO sintase também apresentam resistência à insulina e hiperglicemia (Baron *et al.*, 1995; Baron, 1996; Roy, Perreault e Marette, 1998; Higaki *et al.*, 2001). Além disso, estudos clínicos mostram que a hipertensão tem forte associação com a resistência à insulina: cerca de 50% dos pacientes hipertensos têm hiperinsulinemia ou intolerância à glicose, enquanto 80% dos pacientes diabéticos tipo 2 são hipertensos (Zhou, Wang e Yu, 2014). Pacientes hipertensos sem obesidade e diabetes apresentam altas concentrações plasmáticas de glicose após teste de tolerância oral à glicose e elevada glicemia de jejum, que foi positivamente relacionada com a simpatoexcitação (Wang *et al.*, 2015).

A dislipidemia é outra desordem metabólica que tem sido reportada na hipertensão essencial (Sartika *et al.*, 2015). Alguns estudos reportaram a incidência de 50 a 80% de dislipidemia em pacientes hipertensos (O'meara *et al.*, 2004; Sartika *et al.*, 2015). Esta coocorrência implica em intervenções terapêuticas que tratam ambas as condições (Bays *et al.*, 2007; Sartika *et al.*, 2015). A dislipidemia também tem sido observada em ratos SHR (Iritani *et al.*, 1977; Hajri *et al.*, 2001) e hipertensos induzidos por L-NAME (Cardoso *et al.*, 2013) embora não seja consenso (Singer *et al.*, 1979; Navarro *et al.*, 1994).

Alguns estudos também relatam distúrbios da lipólise do tecido adiposo na hipertensão. Neste contexto, pacientes obesos hipertensos apresentaram concentrações de glicerol plasmático mais baixas que os obesos normotensos, em condições basais ou estimulados por noradrenalina, o que sugere a dessensibilização de receptores β-adrenérgicos no tecido adiposo na hipertensão (Townsend e Klein, 1997). Outros trabalhos com ratos SHR também reportaram menor resposta lipolítica à noradrenalina (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988) e ao isoproterenol (Nelson, Shepherd e Spitzer, 1987) quando comparados com o seu controle WKY. Vários

mecanismos foram sugeridos para explicar este fenômeno: alteração da função da proteína ligadora do nucleotídeo guanina (proteína G), defeitos na regulação de enzimas lipolíticas, baixa afinidade dos receptores β-adrenérgicos (Nelson, Shepherd e Spitzer, 1987) pelos hormônios e/ou menor densidade destes receptores na membrana do adipócito do SHR (Chiappe De Cingolani, 1988).

Ou seja, todos estes estudos relatam a ocorrência de alterações metabólicas na hipertensão essencial, as quais assemelham-se às desordens desencadeadas pela reação do organismo ao estresse crônico; no entanto, em todos estes estudo apresentados, não há investigação do ambiente hormonal relacionado aos hormônios clássicos da reação ao estresse nestes modelos de hipertensão. Mais especificamente em relação à resposta lipolítica dos adipócitos de ratos e humanos, todos estes estudos relatam menor lipólise estimulada no hipertenso, sem no entanto se aprofundarem na função e expressão de subpopulações de receptores β-adrenérgicos, assim como dos outros componentes da via lipolítica.

Desta forma, o presente trabalho teve como objetivo investigar as alterações metabólicas desencadeadas na hipertensão essencial, associadas ao ambiente hormonal relacionado ao estresse, além da sensibilidade lipolítica das diferentes subpopulações de β-adrenoceptores de adipócitos epididimais isolados e a expressão de proteínas da via lipolítica do tecido adiposo epididimal e de UCP3 do músculo esquelético, em modelos de hipertensão. Para isso, 2 modelos de ratos hipertensos foram empregados: os ratos hipertensos induzidos por administração oral crônica de L-NAME e os ratos espontaneamente hipertensos (SHR), com seus repectivos controles Wistar e Wistar-Kyoto (WKY). A escolha destes modelos foi embasada nos seguintes fatores: ambos são extensivamente estudados e utilizados para se compreender a hipertensão essencial no homem; são modelos que apresentam a simpatoexcitação (Bergamaschi, Campos e Lopes, 1999; Dickinson, 2007; Kishi e Hirooka, 2013; Thomas e Dasgupta, 2015) e a desregulação do eixo HPA (Weidenfeld *et al.*, 1999; Gadek-Michalska e Bugajski, 2008) bem estabelecidas na literatura, os quais aparecem independentemente de agentes estressores externos bem estabelecidos; e finalmente o modelo por indução representa a hipertensão adquirida no ambiente e o modelo de hipertensão espontânea, a hipertensão desenvolvida por fatores genéticos.

Objetivos gerais

Avaliar, sob as mesmas condições padrão, dois modelos de hipertensão (induzida por administração oral crônica de L-NAME e a genética), quanto à:

- Hormônios relacionados ao estresse;

- Parâmetros do metabolismo energético;

- Sensibilidade das subpopulações de β-adrenoceptores de adipócitos isolados;

- Expressão de proteínas da via lipolítica e receptores de adenosina e glicocorticoides do tecido adiposo e de UCP3 no músculo esquelético.

Objetivos específicos

Avaliar, sob as mesmas condições padrão, dois modelos de hipertensão: induzida pela ingestão de L-NAME (representado pelos ratos Wistar que ingeriram cronicamente L-NAME na água de beber) e a genética (representado pelos ratos espontaneamente hipertensos ou SHR), juntamente com seus grupos controle, Wistar e Wistar Kyoto, respectivamente, quanto a:

- Hormônios relacionados ao estresse, por meio da mensuração de catecolaminas, corticosterona e ACTH no soro destes animais;

- Parâmetros do metabolismo energético: evolução ponderal, e também relativa ao consumo hídrico e de ração, taxa metabólica, peso dos panículos adiposos (epididimal e retroperitoneal), morfometria dos seus adipócitos e expressão da proteína UCP3 no músculo gastrocnêmio;

- Sensibilidade das subpopupações de β-adrenoceptores de adipócitos isolados da região epididimal, a agonistas β-adrenérgicos (isoproterenol e salbutamol), com e sem antagonistas (metoprolol, propranolol, ICI 118,551 e cafeína);

- Expressão de proteínas da via lipolítica do tecido adiposo epididimal (receptores β-adrenérgicos β_1, β_2 e β_3, proteínas G inibitória (Gi) e estimulatória (Gs), proteína quinase A (PKA), perilipinas, lipase hormônio sensível (HSL), lipase de triacilglicerol (ATGL), proteína do gene comparativo de identificação-58 (CGI-58), receptor de glicocorticoide (GR) e receptores de adenosina A1 e A2.

Para a apresentação desta tese, a metodologia e os resultados foram apresentados nos manuscritos gerados durante o seu desenvolvimento, os quais passamos a apresentar.

Manuscritos

Energy Metabolism and Stress Hormones in two Models of Hypertension: L-NAME-Induced and Spontaneously Hypertensive Rats

Running title: Energy Metabolism and Stress Hormones in Hypertensive Rats

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Conflict of Interest Statement

All authors don`t have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Abstract

Essential hypertension is the most common cardiovascular disease. Stress response is composed by two major arms: activation of sympathetic system and hypothalamus-pituitary-adrenal axis. Besides, these mechanisms seem to be central in essential hypertension too. Some metabolic disturbances have been observed in essential hypertension, and may be due to stress hormones. So the aim of this study was the characterization of stress hormones and some parameters of energy metabolism of N^G-nitro-L-arginine methyl ester (L-NAME)-induced model of hypertension and genetic model of hypertension of Spontaneously Hypertensive rats (SHR). Besides, we compared controls of both models, because Wistar Kyoto rats (WKY) displays raised levels of stress hormones but doesn't develop hypertension, and we also compared both hypertensive groups. We induced hypertension in 10-week-old Wistar rats by chronic oral administration of L-NAME in tap water (40 mg/kg per day) for 5 weeks (L-NAME group). Blood and tissue sampling and euthanasia were performed in 15-week-old rats. Raised levels of catecholamines and corticosterone were observed in L-NAME, WKY and SHR groups, but not in Wistar control rats (W group). L-NAME exhibited metabolic alterations of acute stress response, like reduced adiposity and food intake. WKY also displayed these changes if compared to Wistar, in addition to others, found in chronic stress, like reduced resting metabolic rate and expression of uncoupling protein 3. SHR showed some metabolic disturbances which can be related to hyperfunction of thyroid, like reduced adiposity and elevated food intake and resting metabolic rate, that may have overlapped the effects of stress hormones. These observations suggest the presence of an environment of stress hormones in hypertensive subjects that can be related to disturbances in energy metabolism.

Key-words: hypertension, stress, SHR, WKY, L-NAME-induced hypertension

Introduction

Hypertension is the most common cardiovascular disease, accounting for 9.4 million deaths worldwide every year (Oms, 2013). Essential or primary hypertension is the high blood pressure without secondary causes and it accounts for 95% of all cases of hypertension (Wang *et al.*, 2015).

Many animal models have been used to understand the cause and progression of hypertension as well as therapeutic interventions (Dornas e Silva, 2011). Among them, the hypertension by chronic inhibition of nitric oxide (NO), caused by chronic oral administration of N^G-nitro-L-arginine methyl ester (L-NAME), is characterized by intense peripheral vasoconstriction (Bergamaschi, Campos e Lopes, 1999; Thomas e Dasgupta, 2015) and has been used to mimic hypertension in humans, since NO deficiency is one of the causes of essential hypertension (Berkban *et al.*, 2015). The genetic model of hypertension represented by Spontaneously Hypertensive Rats (SHR) and its Wistar Kyoto (WKY) control is also extensively used because it has a lot of similarities with essential hypertension in humans (Trippodo e Frohlich, 1981; Dornas e Silva, 2011).

Chronic stress is a major risk factor for essential hypertension (Oms, 2013). Stress response is composed by two major arms: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), that are triggered by stressor stimuli to restore allostasis (Mcewen e Gianaros, 2011). According to many studies, chronic stress predisposes to hypertension due to activation of HPA axis (Gold *et al.*, 2005; Goodwin e Geller, 2012) and SNS (Björntorp *et al.*, 2000; Hering e Schlaich, 2015; Thomas e Dasgupta, 2015); the latter considered as the major mechanism.

Besides, chronic activation of SNS and HPA seems to be the central mechanism in essential hypertension, regardless of a well-defined external stressor (Björntorp *et al.*, 2000; Gold *et al.*, 2005; Hering e Schlaich, 2015). This phenomenon can be triggered by small but prolonged elevations in blood pressure which can disturb the neural autonomic balance, causing sympathoexcitation (Hering e Schlaich, 2015), which, in turn, activates HPA axis, due to their several interconnection at different levels (Björntorp *et al.*, 2000).

L-NAME-induced hypertensive rats and SHR rats are two models of hypertension, which have frequently displayed increased sympathetic tone (Bergamaschi, Campos e Lopes, 1999; Dickinson, 2007; Kishi e Hirooka, 2013; Thomas e Dasgupta, 2015) and HPA axis activation (Weidenfeld *et al.*, 1999; Gadek-Michalska e Bugajski, 2008). Besides, the WKY rats have also been associated with HPA activation, because they display raised basal plasma

corticosterone and adrenocorticotropic hormone (ACTH) levels compared to Wistar; so they are called “hyper-reactive” to stress (Will, Aird e Redei, 2003), although normotensive.

There are many studies in the literature about metabolic disturbances causing hypertension, like the so-called “obesity-related hypertension” (Dorresteijn, Visseren e Spiering, 2012; Kalil e Haynes, 2012; Hall *et al.*, 2015). However, there are few studies reporting the inverse relationship, in which hypertension gives rise to metabolic alterations; the majority of them observes the same disturbances triggered by chronic stress (Julius, Valentini e Palatini, 2000; Ferris e Kahn, 2012; Fardet e Fève, 2014; Lee *et al.*, 2014; Thorp e Schlaich, 2015). Some studies showed some metabolic disturbances in hypertensive human and animal models of hypertension, like impaired glucose metabolism (Hulman, Falkner e Chen, 1991; Reaven e Chang, 1991; Baron *et al.*, 1995; Baron, 1996; Roy, Perreault e Marette, 1998; Hajri *et al.*, 2001; Higaki *et al.*, 2001; Potenza *et al.*, 2005; Potenza *et al.*, 2006; Zhou, Wang e Yu, 2014; Wang *et al.*, 2015), obesity (Kannel *et al.*, 1967; Wassertheil-Smoller *et al.*, 1992; Lasser *et al.*, 1995; Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011; Rekleiti, 2014) and dyslipidemia (Iritani *et al.*, 1977; Hajri *et al.*, 2001; O'meara *et al.*, 2004; Cardoso *et al.*, 2013; Sartika *et al.*, 2015); if they are related to stress hormones, it remains to be elucidated.

Besides, stress response is implicated in many other metabolic alterations, like on adiposity, appetite and energy expenditure. There are antagonist responses related to these parameters, depending on the acute or chronic duration of stress response.

In the acute stress, glucocorticoids and catecholamines act synergistically to evoke a lipolytic response to ensure sufficient energy supply for demands required during stress response (Sacta, Chinenov e Rogatsky, 2015), which can reduce adiposity. In this context, there is also appetite suppression and raised energy expenditure triggered by corticotropin-releasing hormone (CRH), which is produced by hypothalamus and initiates HPA axis activation (Nicolaides, Charmandari e Chrousos, 2015). Besides CRH, catecholamines also inhibits appetite and enhances energy expenditure (Messina *et al.*, 2013).

On the other hand, in chronic stress, glucocorticoids effects become dominant and predispose to obesity. Thus, glucocorticoids stimulate lipogenesis and adipogenesis, mainly in visceral adipose tissue (Fardet e Fève, 2014; Lee *et al.*, 2014). They also increase appetite, especially for high-calorie and fatty foods and decrease energy expenditure (Fardet e Fève, 2014). The latter effect may be due to reduction in the expression and activity of uncoupling proteins (UCPs) throughout the body, among other mechanisms (Zakrzewska *et al.*, 1999).

However, the investigation of these parameters in the hypertensive status, and their relation to stress hormones remain to be determined. So the aim of the present study was the characterization of these metabolic parameters and stress hormones in two models of hypertension. We chose the L-NAME-induced hypertensive and SHR rats as models of acquired and genetic hypertension respectively, which have displayed increased sympathetic tone and HPA axis activation. We also performed comparisons between both controls to evaluate the effects of SNS and HPA axis activation in WKY dissociated from hypertension, and we compared both hypertensive groups to evaluate differences between genetic and induced hypertension.

Methods

Animals

Animal procedures were approved by the Committee for Ethics in Animal Experimentation of the Institute of Biology (UNICAMP, 2616-1), and all efforts were made to minimize suffering. All animals were provided by Multidisciplinary Center for Biological Research (CEMIB - UNICAMP). We used two models of hypertension (genetic and induced hypertension), resulting in 4 experimental groups: the model of induced hypertension was composed by normotensive Wistar (HanUnib:WH) rats (W, n=6) and L-NAME-induced hypertensive Wistar (HanUnib:WH) rats (L-NAME, n=6); the model of genetic hypertension was composed by normotensive Wistar Kyoto (NTacUnib:WKY) rats (WKY, n=6) and Spontaneously Hypertensive rats (SHR/NTacUnib) (SHR, n=6). L-NAME administration was adapted from Paulis et al., 2010 (Paulis *et al.*, 2010). L-NAME was given in tap water to 10-week-old Wistar rats (40 mg/Kg/day) for 5 weeks and its concentration was adjusted to water consumption and weight 3 days a week. The rats were housed in collective cages (3 rats per cage) at 22^{o}C on a 12 h light-dark cycle, with lights on at 06:30 a.m. All animals were given chow and water *ad libitum*. The rats were placed into the same vented chamber at the same time, to eliminate possible variables related to environmental conditions. We used transparent cages; so cages of the same group were placed close together but separated from the others to avoid behavioral influence of rats from the other groups by the mechanism of mirror neurons (Bonini, 2016). Blood samples were extracted at around 9 a.m, from 15-week-old anaesthetized rats which were previously starved for 12-16 h before sacrifice. After blood sampling, tissues were excised under anesthetic overload. The rats were anaesthetized with Zoletil 50® (tiletamine, 29 mg/kg and zolazepam, 29 mg/kg i.m.; Vibac Laboratories, Carros,

France) and Anasedan® (xylazine, 12,88 mg/kg, i.m.; Sespo Ind. e Com. Ltda, Paulínia, SP, Brazil). They were euthanized by anesthetic overdose.

Chemicals

Bovine serum albumin (fraction V), collagenase (type II), HEPES, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20, glycerol and anti-α-tubulin (α-tubulin, T5168 mouse monoclonal, registered at Antibody Registry: http://antibodyregistry.org/search?q=T5168) were from Sigma-Aldrich (St Louis, MO, USA). Nitrocellulose membrane (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Kit for corticosterone and ACTH quantification was obtained from Millipore (Billerica, MA). Anti-UCP3 (UCP3, sc-7756 goat polyclonal, registered at Antibody Registry: http://antibodyregistry.org/search?q=sc-7756l) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Both antibodies used in this study were validated by previous studies (Alberdi *et al.*, 2013; Shimasaki *et al.*, 2013; Gómez-Villafuertes *et al.*, 2015; Pazos *et al.*, 2015).

Body weight, food and water intake

Body weights were measured twice a week for 8 weeks, starting at 7 weeks of age. We also assessed wet weight of the epididymal (EFP) and retroperitoneal (RFP) fat pads when they were extracted from overload anesthetized animals. Food and water intake was measured twice a week in the same period of body weight measurements, by providing a fixed amount of chow and water and measuring the unconsumed food and water, dividing them per animals in cage (3 in each cage). Food and water intake was normalized to body weight due to differences in weight of rats from different groups.

Resting metabolic rate

Resting metabolic rate (RMR) was obtained by a dual head space analyzer (MOCON PAC CHECK, model 650), linked to a hermetically sealed respirometer containing the animal. This equipment measured the internal air package composition (oxygen and carbon dioxide concentration), which was obtained per minute, during 4 minutes. RMR was always measured at around 15:00 h in fed animals. RMR was calculated according to the formula: CO_2 production (%) /O_2 consumption (%) x 20292.4 (J) x /body weight (g) / 4 (min). RMR was done twice: at the beginning (6-7 week-old rats) and at the final of the experiments (14-week-old rats).

Measurement of blood pressure

Measurements of arterial blood pressure were recorded from a catheter introduced in the right carotid artery of 15-week-old anesthetized rats by Power Lab 8/30 and they were analyzed using associated software (LabChart Pro, ADInstruments – Australia). The technique was made according Conceição-Vertamatti (2015) (Conceição-Vertamatti *et al.*, 2015). A separate group of animals was used only for this test. This group lived in the same vented chamber as the other rats, at the same time.

Serum analysis

Blood samples were taken out from anesthetized rats by cardiac puncture, before sacrifice. The serum was obtained by centrifugation (10,000 rpm, 15 min, 4^{o} C), placed in a vial and frozen until quantification. Serum corticosterone and ACTH were measured through Luminex 200 (Millipore, Billerica, MA). Serum catecholamines were quantified fluorometrically (excitation 420 nm, emission 510 nm) according to Kelner et al., (1985) (Kelner *et al.*, 1985).

Isolation and morphometry of adipocytes

Adipocytes were isolated from the epididymal, retroperitoneal and mesenteric fat pads, by a modification of the Rodbell's original procedure (Rodbell, 1964; Crege *et al.*, 2014). Morphometry was performed according Crege et al. (Crege *et al.*, 2014). 1-1.5 g of fat pad was fragmented and digested with 1 mg/mL collagenase (type II, from *Clostridium histoliticum*) (Sigma-Aldrich, St Louis, MO, USA), in polyethylene tubes with 3 mL of Krebs-Ringer bicarbonate buffer containing Hepes (25 mM) (Sigma-Aldrich, St Louis, MO, USA), glucose (6 mM), and bovine albumin (3%, BSA fraction V fatty-acid free) (Sigma-Aldrich, St Louis, MO, USA), pH 7.4 (KRBA), at 37^{o}C with shaking (60 cycles/min) during 45 min. The isolated fat cells were filtered through a nylon mesh and washed 3 times with 3 mL KRBA buffer (3% BSA). The final volume of cellular suspension was adjusted to 5 mL with KRBA buffer (3% BSA). 100 μL of cellular suspension were adjusted with KRBA to a 10% suspension: 10 μL of this suspension were transferred to a Mallassez chamber for adipocyte measurement using the IMAGE PRO PLUS ANALYSER software (Media Cybernetics, Silver Spring, MD) after image capture via Leica microscope (632 μm of measured area). This 10% cellular suspension was made once per sample of adipose tissue. 9 images were obtained per area of Mallassez chamber, at 10 x objective magnification.

Western Blotting

Fragments of gastrocnemius muscle (100 mg) were extracted from overload anesthetized rats and were immediately frozen in liquid nitrogen, after which they were stored in biofreezer (-80 ^{o}C). They were thawed and homogenized in solubilization buffer at 4 ^{o}C 1% Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/mL with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Homogenized issue was centrifuged for 40 min at 11,000 rpm in a 70.Ti rotor (Beckman) at 4^{o}C to remove insoluble material. Protein concentration of supernatants was obtained by Bradford. Aliquots of the supernatants containing 0.050 mg of protein extracts were separated by SDS PAGE, transferred to nitrocellulose membranes and blotted with antibodies. Membranes were incubated with 10 mL of 3% BSA solution containing 1 μL of α-tubulin antibody or 15 μL of UCP3 antibody. Specific bands were detected by chemiluminescence and capture was performed with a Syngene GBox (Imgen Technologies, Alexandria, VA, USA). Densitometry analysis by UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA) was used to quantify protein expression, whose pixel intensities were normalized to α−tubulin, which were normalized to mean values of control groups (W or WKY). Details about each assay were presented at figure in Supplementary figure 1.

Statistical analysis

The values are shown as means $\pm$ s.e.m. The normality was confirmed by Kolmogorov-Smirnov test and then we performed unpaired Student's t-test for comparisons between two groups and paired Student's t-test for comparisons "before-after" within each group (W, L-NAME, WKY or SHR). In addition to "before-after" analysis, we quantified these changes from 7 to 14 weeks of age, considering the allometric influence of body size on food intake and metabolic rate (Tschöp *et al.*, 2012). All statistical analysis were done with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered significant for $p<0.05$.

Results

Arterial blood pressure

At the end of experiment, mean blood pressure was higher in hypertensive groups compared to their respective controls (W: 106.9 ± 9.2 mm Hg *versus* L-NAME: 145.2 ± 12.4 mm Hg; WKY: 107.1 ± 5.9 mm Hg *versus* SHR: 127.1 ± 4.9 mm Hg, $p<0.05$). No differences were found between both control groups and between both hypertensive ones.

Body weight, food and water intake and resting metabolic rate

These parameters were analyzed from two perspectives: the changes within each group from 7 to 14 weeks of age and the differences normalized to body weight between groups at 7 and 14 weeks of age. The longitudinal analysis within each group showed that W and L-NAME displayed the same changes in body weight gain (Fig, 1A and 2A), in the reduction of food intake (Fig, 1B and 2C) and of RMR (Fig. 2E). However, SHR exhibited increased weight gain (Fig. 1A and 2A) and greater reduction of food intake (Fig. 1B and 2C) and RMR (Fig. 2E) than WKY. Besides, WKY displayed decreased weight gain (Fig, 1A and 2A) and less reduction of food intake (Fig. 1B and 2C) than W, while no differences were found in the reduction of RMR (Fig. 2E). Finally, L-NAME and SHR groups showed the same weight gain (Fig. 1A and 2A); however SHR presented lower reduction of food intake (Fig. 1B and 2C) along with greater reduction of RMR than L-NAME (Fig. 2E).

The analysis of parameters normalized to body weight between groups showed no differences in body weight (Fig. 1B) and RMR (Fig. 1F) between W and L-NAME group at both 7 and 14 weeks of age. However, L-NAME consumed less amounts of food than W at 14 weeks of age, while at 7, food intake didn't differ between groups (Fig. 1D). On the other hand, SHR displayed lower values of body weight (Fig. 1B) and elevated food intake (Fig. 1D) and RMR (Fig. 1F) than WKY and L-NAME at both ages. Body weight of WKY was greater than W from beginning to the end (Fig. 1B); however WKY had lower food intake (Fig. 1D) with the same RMR initially (Fig. 1F), whereas its food intake was not different (Fig. 1D) and RMR was lower than W, at the final (Fig. 1F).

Water intake was not different between W and L-NAME at 7 weeks of age (W: 0.12 ± 0.005 mL/g *vs* L-NAME: 0.14 ± 0.01 mL/g); however L-NAME increased its water consumption at 14 weeks of age (W: 0.09 ± 0.001 mL/g *vs* L-NAME: 0.11 ± 0.002 mL/g). There were no differences at the final water intake between WKY and SHR **(**WKY: 0.12 ± 0.004 mL/g *vs* SHR: 0.12 ± 0.008 mL/g**).** WKY exhibited greater water intake than W at 7 **(**W: 0.12 ± 0.005 mL/g *vs* WKY: 0.16 ± 0.003 mL/g) and 14 **(**W: 0.09 ± 0.001 mL/g *vs* WKY: 0.12 ± 0.004 mL/g) weeks of age. At 7 weeks of age, SHR consumed more amounts of water than L-NAME (L-NAME: 0.14 ± 0.01 mL/g *vs* SHR: 0.17 ± 0.005), however, there were no

differences in the water intake at 14 weeks of age (L-NAME: 0.11 ± 0.002 mL/g *vs* SHR: 0.12 ± 0.008 mL/g).

Serum parameters

We observed a hormonal environment of stress response in L-NAME and WKY which displayed raised levels of catecholamines and corticosterone compared to W. On the other hand, raised levels of hormones of stress response were also present in SHR, which didn't differ from WKY in catecholamines and corticosterone and showed lower serum ACTH than WKY. Besides, L-NAME and SHR did not differ in corticosterone and ACTH levels, but SHR displayed lower levels of catecholamines than L-NAME (Table 1).

Table 1. Serum catecholamines, corticosterone and ACTH of blood samples collected at around 9 a.m. from 12-16 h fasted 15-week-old anesthetized rats from W (n=5-6), L-NAME (n=6), WKY (n=6) and SHR (n=6) group.

	W	**L-NAME**	**WKY**	**SHR**
Catecholamines (μM)	45.1 ± 2.4	65.2 ± 2.4[a]	64.8 ± 4.6[c]	59.5 ± 1.6[d]
Corticosterone (pg/mL)	31570 ± 7175	73490 ± 19320[a]	78200 ± 10370[c]	81890 ± 4017
ACTH (pg/mL)	0.8 ± 0.1	1.0 ± 0.2	5.1 ± 1.2[c]	1.1 ± 0.1[b]

Data expressed as mean ± s.e.m.

[a] W *versus* L-NAME; [b] WKY *versus* SHR; [c] W *versus* WKY; [d] L-NAME *versus* SHR (Student's unpaired t-test, $p<0.05$).

Fat pad weight and adipocyte size

No differences were found in EFP weight/body weight between W and L-NAME, and between W and WKY, whereas it was decreased in SHR when compared to WKY and L-NAME. There were no differences in adipocyte area of EFP among groups except in SHR, which presented smaller fat cells than WKY. Hypertensive groups showed decreased RFP weight/body weight and smaller RFP adipocyte size when compared to their controls; when both controls were compared to each other, SHR showed diminished RFP weight/body weight compared to L-NAME, but there was no difference in RFP adipocyte size. WKY presented decreased relative RFP weight/body weight compared to W and this comparison was not different concerning RFP adipocyte size. Both hypertensive groups also presented lower values of mesenteric adipocyte size than their controls while this parameter was lower in WKY compared to W and in SHR compared to L-NAME (Table 2, Fig. 3).

Table 2: Relative weight of epididymal and retroperitoneal fat depot and area of adipocytes isolated from epididymal, retroperitoneal and mesenteric fat pads of 12-16 h fasted 15-week-old rats from W (n=5-6), L-NAME (n=5-6), WKY (n=6) and SHR (n=5-6) group under anesthetic overload.

	W	**L-NAME**	**WKY**	**SHR**
Relative EFP (mg/g)	17.2 ± 1	16.4 ± 1.5	19.1 ± 1	9.1 ± 0.6[b d]
Relative RFP (mg/g)	24.1 ± 2.3	17.1 ± 2.4[a]	16.9 ± 1.2[c]	12.1 ± 1[b d]
Epididymal adipocyte area (μm^2)	4848 ± 531	3783 ± 357	4621 ± 360	3141 ± 232[b]
Retroperitoneal adipocyte area (μm^2)	4798 ± 371	3412 ± 295[a]	5105 ± 681	3623 ± 326[b]
Mesenteric adipocyte area (μm^2)	3206 ± 252	2373 ± 77[a]	2691 ± 140[c]	1997 ± 47[b d]

Data expressed as mean ± s.e.m.

EFP, epididymal fat depot; RFP, retroperitoneal fat depot.

[a] W *versus* L-NAME; [b] WKY *versus* SHR; [c] W *versus* WKY; [d] L-NAME *versus* SHR, (Student's unpaired t-test, $p<0.05$).

Western Blot analysis

There were no differences in uncoupling protein 3 (UCP3) expression between hypertensive groups (L-NAME and SHR) and their respective controls (W and WKY) (Fig. 4 A,B). However, WKY displayed lower UCP3 expression than W group (Fig. 4 C).

Discussion

We conducted the discussion into two different perspectives: first, we discussed the changes in parameters measured at 7 and 14 weeks of age considering allometry; later, we associated differences between groups with serum and molecular data, in each comparison.

According to allometry, some metabolic parameters, like food consumption and metabolic rate, do not vary linearly with body size because the greater surface-to-weight ratio, the greater heat loss to the environment, which requires more energy expenditure to maintain body temperature (Tschöp *et al.*, 2012). So W and L-NAME showed the same weight gain,

which reduced their surface-to-weight ratio in the same proportion, causing the same reduction in food intake and RMR. On the other hand, SHR presented greater weight gain than WKY; it caused higher reduction of surface-to-weight ratio which reduced its energy expenditure to maintain body temperature, decreasing energy demands and food ingestion, if compared to WKY. In the same way, WKY displayed less weight gain than W, and consenquently less reduction of food intake, but not of RMR, probably due to its decreased value in WKY group compared to W at the final, which will be better clarified bellow. Besides, L-NAME and SHR showed the same weight gain, but SHR displayed lower reduction of food intake, which can be related to its high consumption of food at the final, compared to L-NAME, further explored bellow, in the comparisons made specifically at 7 and 14 weeks of age. Despite the greater weight gain and higher reduction of food intake and RMR exhibited by SHR, it showed lower body weight and higher food intake and RMR than WKY and L-NAME at 7 and 14 weeks of age, as we will discuss bellow.

In the induced model of hypertension, we identified raised levels of stress hormones, catecholamines and corticosterone compared to its control, W. These hormones can explain some metabolic alterations in L-NAME. The reduced food intake of L-NAME group at 14 weeks of age, could be evoked by corticotropin-releasing hormone (CRH), which is produced by hypothalamus and initiates HPA axis activation. CRH stimulates anorexigenic neurons in arcuate nucleus (ARC) of hypothalamus, that produce pro-opiomelanocortin (POMC), which originates alpha-melanocyte-stimulating hormone (α-MSH), that decreases food intake (Crespo *et al.*, 2014). It is a hypophagic response to stress, observed in rats under acute stress (Maniscalco *et al.*, 2015). Although we didn't measure CRH levels, we hypothesized that it affected food intake, because of the activation of HPA axis demonstrated by high levels of corticosterone.

Decreased RFP weight and adipocyte size of retroperitoneal and mesenteric fat pads can be attributed to low food consumption and the lipolytic effects of catecholamines (Thorp e Schlaich, 2015) and corticosterone (Wang *et al.*, 2012). Both hormones trigger lipolysis under acute stress, but catecholamines can cause desensitization of β-adrenoceptors (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011) and corticosterone can induce lipogenesis in visceral adipose tissue (Fardet e Fève, 2014) under chronic stress. Therefore L-NAME group displayed some metabolic alterations of acute response to stress, despite of its chronic oral ingestion of L-NAME.

L-NAME treatment clearly evoked increased water intake. Although we did not measured circulating angiotensin II and aldosterone, the activation of renin-angiotensin-

aldosterone system (RAAS) is well described in L-NAME treated rats (Takemoto *et al.*, 1997; Ikeda *et al.*, 2009; Suehiro *et al.*, 2015) and can also be explained by stress hormones, in particular, chronic exposure to catecholamines that activate RAAS (Kishi e Hirooka, 2013). Thus we suggest that angiotensin II stimulated thirst centers in the brain of rats from L-NAME group (Bezalel *et al.*, 2015), and aldosterone increased sodium reabsorption (Rossier, Baker e Studer, 2015), which may have contributed to thirst.

Interestingly, some metabolic alterations in L-NAME group attributed to stress hormones can also be found in WKY group, in the comparison W *versus* WKY, because this group also displayed raised catecholamines and corticosterone levels than W, in addition to high levels of ACTH, but did not develop hypertension. Besides, raised ACTH levels in WKY have been attributed to defective HPA axis function due to decreased sensitivity to glucocorticoids, which may decrease their action on the negative feedback in pituitary gland (Solberg *et al.*, 2001).

Although WKY showed higher body weight than W at both ages, WKY consumed less food initially, but presented the same consumption at the final. We suggest that the first is a hypophagic response to stress, the same manner as L-NAME, which is elicited by CRH. However increased food intake that become equivalent to W consumption and the reduction of RMR is consistent with corticosterone effect, which inhibits CRH production and stimulates neuropeptide Y (NPY) synthesis in hypothalamus, which increases appetite and diminishes energy expenditure (Crespo *et al.*, 2014). It's a hyperphagic response to chronic stress, which succeeds hypophagic response of acute stress (Yau e Potenza, 2013). It probably occurred due to greater period of glucocorticoid exposure in WKY than in L-NAME.

Decreased expression of uncoupling protein 3 corroborates the reduced RMR, due to its major effect on thermogenesis of skeletal muscle and thus on the energy expenditure (Depieri *et al.*, 2004). Accumulating evidences from studies with rodents suggest that prolonged exposure to catecholamines (Depieri *et al.*, 2004) and central action of glucocorticoids chronically (Zakrzewska *et al.*, 1999) can produce a marked decrease in UCP3 expression. So we suggest that the metabolic alterations observed in WKY are due to the effects of prolonged exposure to stress hormones, compared to L-NAME group. Despite these differences, we also observed reduced adiposity in WKY compared to W probably triggered by the lipolytic effects of catecholamines (Thorp e Schlaich, 2015), corticosterone (Wang *et al.*, 2012) and ACTH (Chaves, Frasson e Kawashita, 2011) and elevated water ingestion by WKY, probably triggered by raised levels of angiotensin II (Kishi e Hirooka, 2013), and aldosterone (Rossier, Baker e Studer, 2015) the same manner as L-NAME.

On the other hand, in the genetic model of hypertension, SHR did not differ from WKY in the levels of stress hormones, catecholamines and corticosterone. However, ACTH was decreased in SHR rats compared to WKY. It is consistent with the defective HPA axis function of WKY, in which glucocorticoids exert a diminished inhibition of ACTH production due to decreased sensitivity of pituitary gland to glucocorticoids (Solberg *et al.*, 2001), as already mentioned. Besides, some studies also report disturbances in HPA axis in SHR rats (Gómez, De Kloet e Armario, 1998; Djordjevic *et al.*, 2007), presenting lower levels of ACTH (Gómez, De Kloet e Armario, 1998). Thus, metabolic differences between WKY and SHR cannot be attributed solely to stress hormones, since they don't differ between groups, except ACTH.

So another endocrine disorder may explain these differences between both groups: the thyroid hyperfunction in SHR (Wright *et al.*, 1978; Heckmann e Zimmer, 1992). The hyperfunction of thyroid gland can also be triggered by stress hormones through sympatoexcitation in paravenricular nucleus of the hypothalamus, which produces thyrotropin-releasing hormone (TRH), that initiates the hypothalamus-pituitary-thyroid (HPT) axis (Bruhn e Jackson, 1992). It has been shown that thyroid hormones are involved in the modulation of blood pressure and in the development of cardiac hypertrophy in SHR rats and that thyroidectomy prevented the development of hypertension in SHR (Heckmann e Zimmer, 1992).

Furthermore, excessive thyroid hormone triiodothyronine (T3) is involved in raised metabolic functions, like food and water intake, thermogenesis and metabolic rate (Duntas e Brenta, 2012). So we suggest that enhanced food intake and RMR can be attributed to raised levels of T3. Besides, the reduced adiposity of SHR compared to WKY may be due to increased energy expenditure or even the lipolytic effects of thyrotropin-stimulating hormone (TSH), which has also been reported to be elevated in SHR (Bruhn e Jackson, 1992). T3 up regulates UCP3 expression and stimulates uncoupling activity of UCP3 (Lombardi *et al.*, 2015). Although UCP3 was not different between WKY and SHR, we suggest that T3 acts on UCP3 activity, since its down regulation by central action of glucocorticoids may be a compensating factor (Zakrzewska *et al.*, 1999).

Finally, in the comparison between hypertensive groups, in which both presented the environment of stress hormones, they did not differ in corticosterone and ACTH levels, but SHR displayed lower levels of catecholamines than L-NAME. It suggests that the L-NAME ingestion may have elicited greater SNS activation than triggered by the genetic susceptibility of SHR, in our experimental conditions. Despite the raised levels of catecholamines in L-

NAME, the metabolic differences between hypertensive groups were attributed to thyroid hyperfunction of SHR, the same manner as in WKY *vs* SHR comparison.

Therefore high levels of T3 hormone in SHR can also explain its elevated food intake and RMR compared to L-NAME, at both ages. The lower adiposity of SHR than L-NAME can also be attributed to thyroid hyperfunction, due to higher RMR exhibited by SHR or maybe due to the lipolytic action of TSH, as discussed above, which indicates that this endocrine disturbance may have overlapped the lipolytic effect of high catecholamine levels in L-NAME.

Interestingly, L-NAME and SHR did not differ in water intake at the final, suggesting that both models of hypertension display the same degree of RAAS activation.

In summary, our results observed some metabolic alterations in hypertensive rats and WKY normotensive rats that can be attributed to stress hormones. Although L-NAME group has developed hypertension from chronic ingestion of L-NAME, they displayed metabolic alterations observed in acute stress. These data differ from others, maybe due to the shorter period of induction of hypertension compared to others (Cardoso *et al.*, 2013) or due to the use of strains more susceptible to metabolic alterations, like Sprague Dawley rats (Roy, Perreault e Marette, 1998; Shankar *et al.*, 1998; Higaki *et al.*, 2001). On the other hand, WKY rats showed some metabolic alterations of chronic stress, maybe related to the effects of prolonged exposure to glucocorticoids (Will, Aird e Redei, 2003). A possible limitation of this study is the confounding effects of NO in lipolysis: it is able to facilitate leptin-induced lipolysis and to inhibit catecholamine-induced lipolysis (Frühbeck *et al.*, 2014). One can assume that the reduced adiposity of L-NAME group compared to W may be due to the lack of inhibition of catecholamine-induced lipolysis by NO. However, our hypothesis of the role of the lipolytic effects of stress hormones reducing adiposity is supported by the decreased adiposity also found in WKY compared to W. On the other hand, SHR rats also presented raised stress hormones, however, in this study; their metabolic alterations are largely explained by hyperfunction of thyroid gland, which may be triggered by stress. These observations suggest the presence of an environment of stress hormones in hypertensive subjects that can be related to disturbances in energy metabolism.

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Figure legends

Figure 1. Time course of body weight (A) and food intake (B) of rats from W (n=6), L-NAME (N=5), WKY (N=6) and SHR (n=6) group.

Data plotted as mean ± s.e.m.

Figure 2. Weight gain (A), reduction in food intake (C) and in RMR (E) of week 14 as a percentage of week 7 and body weight (B), food intake (D) and RMR (F) of rats from W (n=6), L-NAME (N=5), WKY (N=6) and SHR (n=6) group at 7 and 14 weeks old.

RMR, resting metabolic rate.

[a] W *versus* L-NAME; [b] WKY *versus* SHR; [c] W *versus* WKY; [d] L-NAME *versus* SHR (Student's unpaired t-test, $p<0.05$).

* Comparison between 7 and 14 weeks of age within the same group (Student's paired t-test, $p<0.05$).

i: initial (7 weeks); f: final (14 weeks).

Figure 3. Micrograph of isolated adipocytes from epididymal (A, D, G), retroperitoneal (B, E, H) and mesenteric (C, F, I) fat pads of 12-16 h fasted 15-week-old rats from W, L-NAME, WKY and SHR group under anesthetic overload.

Bar, 250 μm.

Figure 4. Western Blot analysis showing UCP3 expression of gastrocnemius muscle from induced model of hypertension W (n=4), L-NAME (n=4) (A); genetic model of hypertension WKY (n=4), SHR (n=5) (B) and comparison between both controls W (n=4), WKY (n=4) (C). Protein expression was normalized to α-tubulin, which was normalized to mean values of control (W or WKY). Cropped blots were used. Full-length blots are presented at Supplementary figure 1.

[c] W *versus* WKY (Student's unpaired t-test, $p<0.05$). Data are mean ± s.e.m. (n = 4), $p<0.05$.

Supplementary information captions

Supplementary figure 1. Original gel images of Western Blot for Figure 4 in the main text. UCP3 and α-tubulin were blotted at different membranes, with the same samples, in each comparison (W *vs* L-NAME; WKY *vs* SHR and W *vs* WKY).

Figure 1

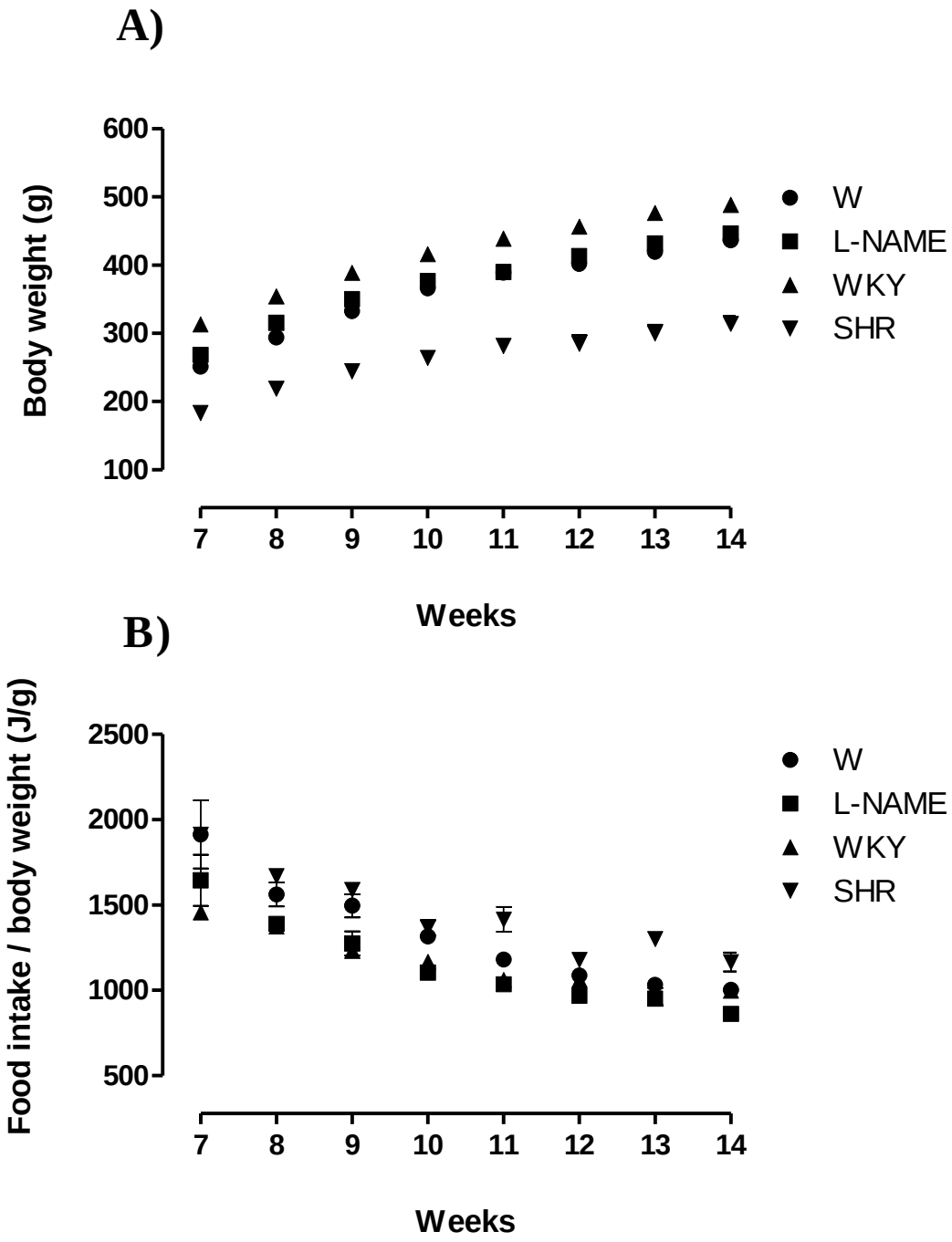


Figure 2

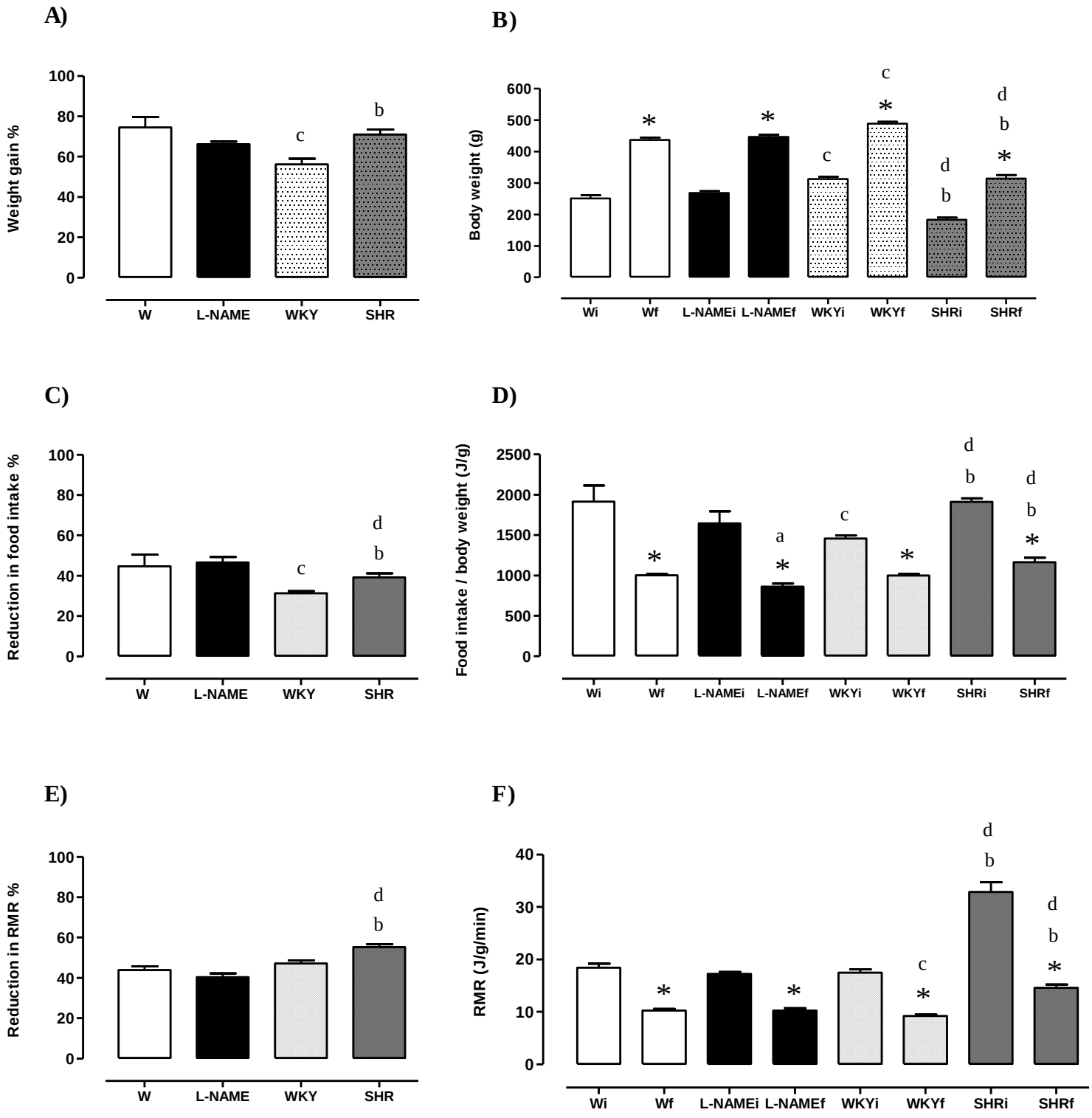

A)
Weight gain %
100
80
60
40
20
0
c
b
W
L-NAME
WKY
SHR
B)
Body weight (g)
600
500
400
300
200
100
0
*
*
*
c
c
d
b
d
b
*
Wi
Wf
L-NAMEi
L-NAMEf
WKYi
WKYf
SHRi
SHRf
C)
Reduction in food intake %
100
80
60
40
20
0
c
d
b
W
L-NAME
WKY
SHR
D)
Food intake / body weight (J/g)
2500
2000
1500
1000
500
0
*
a
*
c
*
d
b
d
b
*
Wi
Wf
L-NAMEi
L-NAMEf
WKYi
WKYf
SHRi
SHRf
E)
Reduction in RMR %
100
80
60
40
20
0
d
b
W
L-NAME
WKY
SHR
F)
RMR (J/g/min)
40
30
20
10
0
*
*
c
*
d
b
d
b
*
Wi
Wf
L-NAMEi
L-NAMEf
WKYi
WKYf
SHRi
SHRf

Figure 3

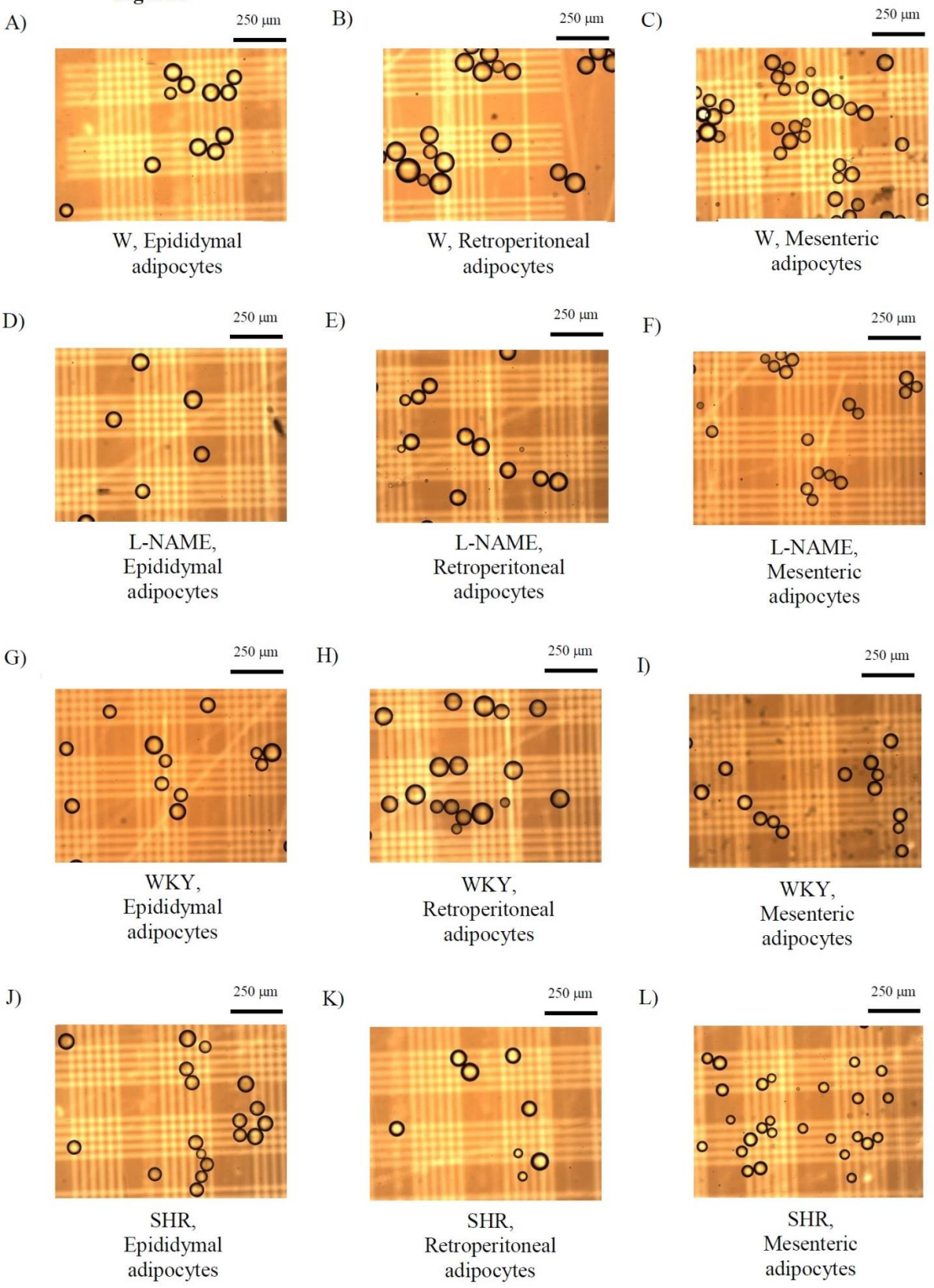
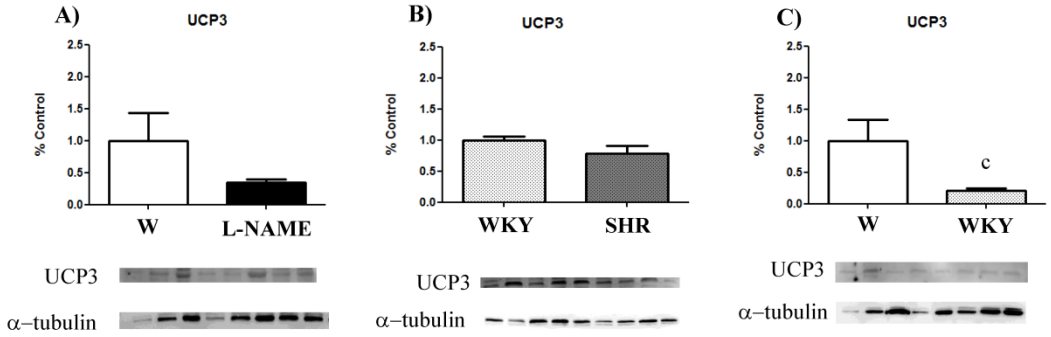
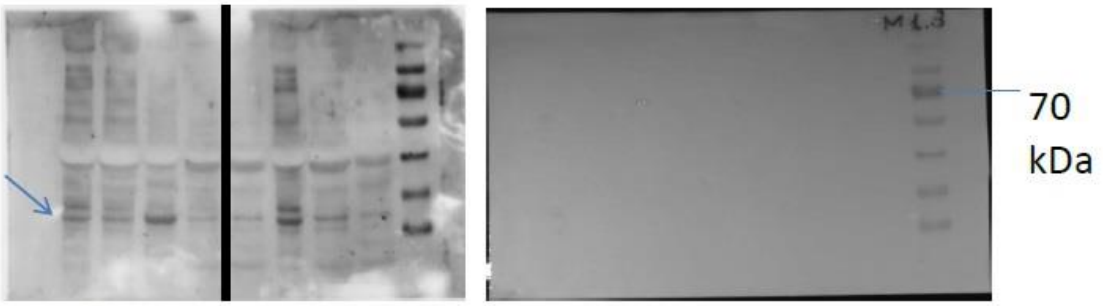

A)
250 µm
W, Epididymal adipocytes
B)
250 µm
W, Retroperitoneal adipocytes
C)
250 µm
W, Mesenteric adipocytes
D)
250 µm
L-NAME, Epididymal adipocytes
E)
250 µm
L-NAME, Retroperitoneal adipocytes
F)
250 µm
L-NAME, Mesenteric adipocytes
G)
250 µm
WKY, Epididymal adipocytes
H)
250 µm
WKY, Retroperitoneal adipocytes
I)
250 µm
WKY, Mesenteric adipocytes
J)
250 µm
SHR, Epididymal adipocytes
K)
250 µm
SHR, Retroperitoneal adipocytes
L)
250 µm
SHR, Mesenteric adipocytes

Figure 4

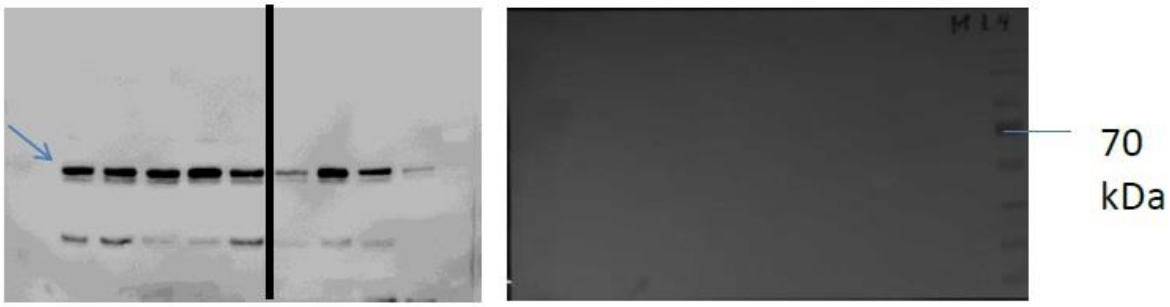

A)
UCP3
% Control
2.5
2.0
1.5
1.0
0.5
0.0
W
L-NAME
UCP3
α–tubulin
B)
UCP3
% Control
2.5
2.0
1.5
1.0
0.5
0.0
WKY
SHR
UCP3
α–tubulin
C)
UCP3
% Control
2.5
2.0
1.5
1.0
0.5
0.0
c
W
WKY
UCP3
α–tubulin

S1 **Figure. Original gel images of Western Blot for Figure 4 in the main text.** UCP3 and α-tubulin were blotted at different membranes, with the same samples, in each comparison (W *vs* L-NAME; WKY *vs* SHR and W *vs* WKY).

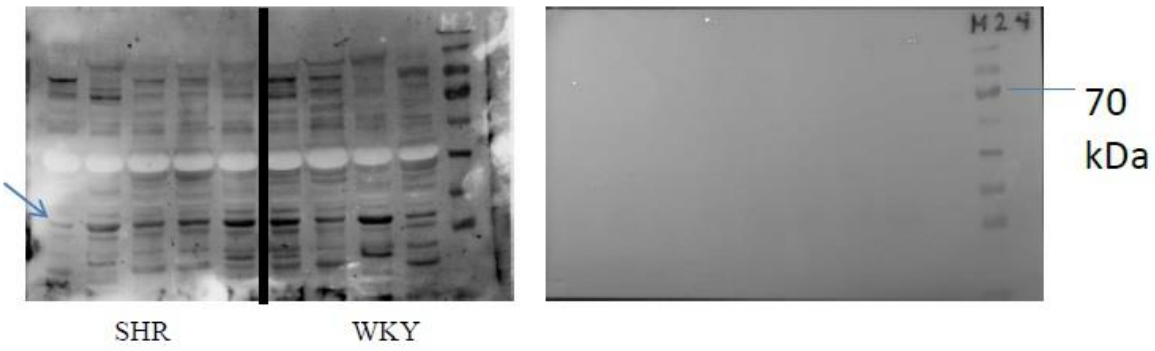
A) UCP3: W *versus* L-NAME. 1 stripping was performed before the blotting with UCP3 antibody



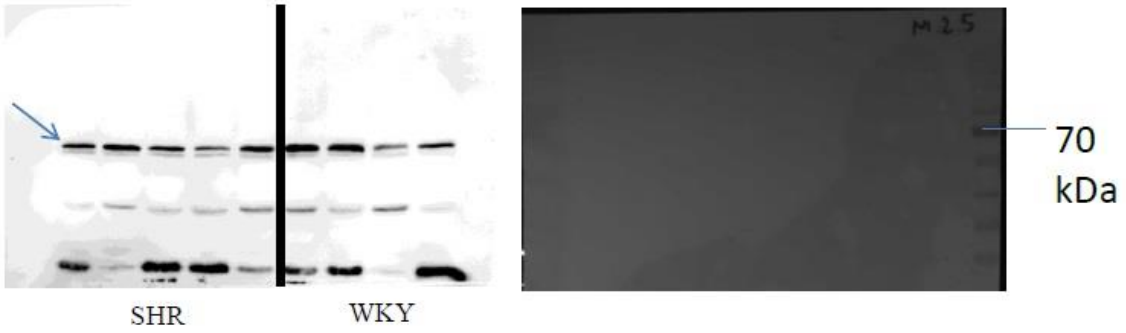
B) α-tubulin: W *versus* L-NAME. 1 stripping was performed before the blotting with α-tubulin antibody



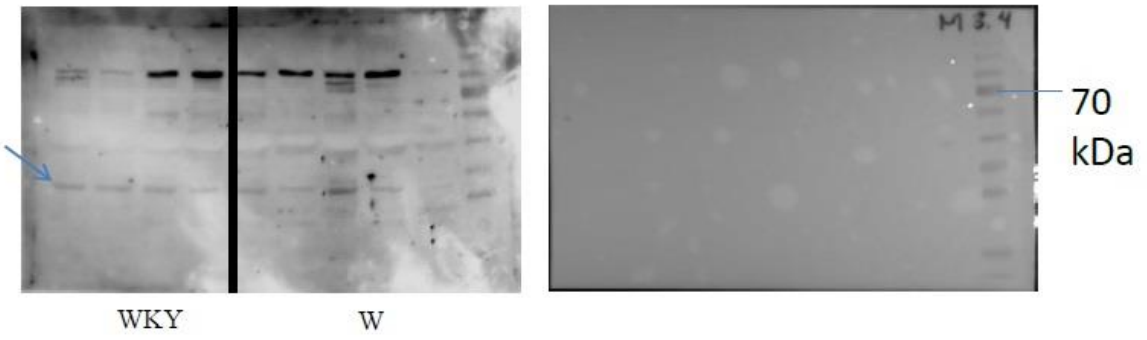
C) UCP3: WKY *versus* SHR. There was no stripping before the blotting with UCP3 antibody



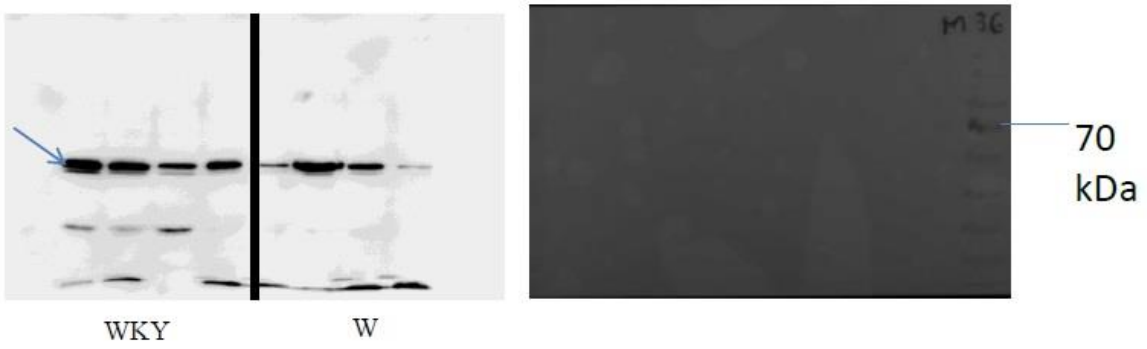
D) α-tubulin: WKY *versus* SHR. 1 stripping was performed before the blotting with α-tubulin antibody



E) UCP3: W *versus* WKY. There was no stripping before the blotting with UCP3 antibody



F) α-tubulin: W *versus* WKY 1 stripping was performed before the blotting with α-tubulin antibody



Alterations in Lipolysis of L-NAME-induced Hypertensive Rats

Running title: Lipolysis in hypertensive rats

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Conflict of Interest Statement

All authors don`t have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Abstract

Stress causes sympathoexcitation, which has been associated with many metabolic disturbances. Previous studies from our lab demonstrated increased β_2-adrenoceptor and decreased β_1- and β_3-adrenoceptor lipolytic responses in isolated adipocytes of rats from acute stress models (swimming and footshock stress). Besides that, studies with human and animal models have associated hypertension with blunted adipocyte lipolysis, which have been attributed to chronic sympathoexcitation in hypertensive subjects. Some rat models of hypertension exhibit sympathoexcitation as the model of nitric oxide system inhibition induced by chronic administration of N^G-nitro-L-arginine methyl ester (L-NAME). Adenosine is able to modulate adrenergic response by two different receptors (A1 and A2), which are expressed in adipocytes. Therefore our aim was to study the sensitivity of β-adrenoceptor subpopulations in isolated epididymal adipocytes as well as the expression of them associated with other proteins of lipolysis pathway in epididymal fat pads of rats treated with L-NAME. 10 week-old Wistar rats were treated with L-NAME in tap water, adjusted to water intake and weight (40 mg/Kg/day), for 5 weeks. Samples of epididymal adipose tissue were extracted from 12-16 h fasting and anesthetized rats, to evaluate protein expression, as well as the lipolytic responses in isolated adipocytes. Lipolytic response was evaluated in isolated adipocytes to isoproterenol and salbutamol in the absence or presence of metoprolol, propranolol, ICI 118, 551 and caffeine. Our molecular results showed a down-regulation of β_1-adrenoceptor, adenosine A2 receptor and perilipin and an up-regulation of HSL. There is an increased β_2-adrenoceptor mediated lipolysis without alterations in β_2-adrenoceptor expression and in any second messengers involved. These functional alterations can raise the importance of adrenaline as an endogenous ligand, thus increasing catecholamine-induced lipolysis, which can contribute to metabolic disturbances associated with hypertension.

Introduction

Stress is a transactional process triggered by real or perceived demands, evaluated as threatening or benign, depending on the adaptive conditions of an individual. It generates biological, behavioral and social coping responses, which influence the risk for and resilience against ill health (Mcewen e Gianaros, 2011). Stress response is composed by two major arms: the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS), that are triggered by stressor stimuli to restore allostasis (Mcewen e Gianaros, 2011). Chronic stress has been associated with disturbances, like on endocrine, neural, immune and cardiovascular system and metabolism (Chandola, Brunner e Marmot, 2006; Matsuura *et al.*, 2015), but little is known of the mechanisms by which stress affects adiposity (Matsuura *et al.*, 2015). Many of these alterations have been attributed to chronic sympathoexcitation, which is suggested to be the main mechanism underlying metabolic syndrome (Thorp e Schlaich, 2015) and its conditions separately (Brooks *et al.*, 2015; Hering e Schlaich, 2015).

Our group has investigated the effects of different acute stress protocols in rat metabolism, focusing in adipose tissue (Farias-Silva *et al.*, 1999; Verago, Grassi-Kassisse e Spadari-Bratfisch, 2001; Farias-Silva *et al.*, 2002; Sampaio-Barros *et al.*, 2003; Farias-Silva *et al.*, 2004). The lipolytic response of white adipocytes to catecholamines is triggered by β-adrenergic receptors (β-ARs). There are 3 subtypes of β-ARs in fat cells: β_1-AR, β_2-AR and β_3-AR, which suggests complex regulation of lipomobilization, governed by different signaling functions (Lafontan, 2012). Under physiologic conditions, catecholamine-induced lipolysis occurs mainly through β_1- and β_2-ARs in human white adipocytes and through β_1- and β_3-ARs in rats (Lafontan, 2012). A role for β_3-ARs in human adipocytes is still under debate, while β_2-ARs represent a small proportion of β-ARs in rat adipocytes (Lafontan, 2012).

Lipolysis can be modulated by different substances, as adenosine. Adenosine is generated from adenosine triphosphate (ATP), and acts through its Gi- and Gs-coupled receptors, that are adenosine A1 and A2, respectively (Koupenova e Ravid, 2013). Adipocytes have both types, but there are more adenosine A1 receptor than A2; so adenosine exerts an inhibitory effect on lipolysis (Panchal *et al.*, 2012; Frühbeck *et al.*, 2014).

Expression of β-adrenoceptors is regulated by catecholamines and glucocorticoids, which are able to cause desensitization or sensitization of each β-AR subpopulation (Lafontan, 2012). We have demonstrated, increased basal lipolysis, decreased β_1- and β_3-ARs mediated response and augmented β_2-AR response in isolated epididymal adipocytes of foot-

shock stressed rats (Farias-Silva *et al.*, 1999) and of swimming stressed rats (Sampaio-Barros *et al.*, 2003; Farias-Silva *et al.*, 2004).

Hypertension is the most common cardiovascular disease and remains as a great health global problem, with great impact on cardiovascular morbidity and mortality (Hering e Schlaich, 2015). Hypertension has multifactorial origin and chronic stress is one of the most relevant which is involved with the origin of this disease (Hering e Schlaich, 2015). While the SNS activation has a well-recognized function in hypertension pathophysiology (Kishi e Hirooka, 2013; Hering e Schlaich, 2015), through α- (arteriolar resistance) and β-adrenoceptors (cardiac output), its role in energy metabolism has been underappreciated (Thorp e Schlaich, 2015).

However some few studies reported metabolic disturbances in hypertension that may be implicated in obesity predisposition. Reduction in blood pressure has been observed in patients who decreased their body weight (Rekleiti, 2014), and hypertensive patients have presented difficulties in losing their body weight (Wassertheil-Smoller *et al.*, 1992; Lasser *et al.*, 1995; Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011). The Framingham Heart Study reported higher risk for the development of obesity in hypertensive subjects comparing to normotensive ones (Kannel *et al.*, 1967). This poorly clarified predisposition is attributed to sympathoexcitation (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011), causing desensitization of β-adrenoceptors, which has been demonstrated *in vivo* and *in vitro* (Hausdorff, Caron e Lefkowitz, 1990; Lohse, 1993; Lambert *et al.*, 2010), and may decrease thermogenesis of brown adipose tissue and skeletal muscle (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011). Besides, studies in human and animal models observed some lipolytic alterations in white adipose tissue under β-adrenergic receptor stimulation in hypertension (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988; Townsend e Klein, 1997), which can be associated with metabolic disturbances in hypertensive subjects. One study (Townsend e Klein, 1997) observed lower levels of circulating glycerol in hypertensive patients in basal conditions or stimulated by adrenaline, when compared to normotensive ones, which supports the idea of desensitization of β-adrenoceptors in hypertension, without further molecular explanation. Other studies with genetic spontaneously hypertensive rats (SHR) reported lower lipolysis induced by noradrenaline (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988) and isoproterenol (Nelson, Shepherd e Spitzer, 1987) when compared to

Wistar Kyoto (WKY) control, but they lack molecular elucidation about the alterations in lipolytic pathway and in β-adrenoceptor subpopulations.

Many animal models have been used to understand the cause and progression of hypertension as well as therapeutic interventions (Dornas e Silva, 2011). Among them, the hypertension by chronic inhibition of nitric oxide, caused by chronic oral administration of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase, is characterized by intense peripheral vasoconstriction and has been associated with sympathetic overactivity of central origin, which exerts an important role in the initiation and the maintenance of hypertension (Bergamaschi, Campos e Lopes, 1999; Thomas e Dasgupta, 2015).

Thus hypertension can alter β-adrenoceptors of adipocytes through chronic sympathoexcitation, and thus can modify lipolysis of white adipose tissue, which can lead to metabolic disturbances. To test this hypothesis, we performed a study with a rat model of hypertension induced by L-NAME, in which we evaluated the lipolysis triggered by two agonists of β-adrenoceptors (non-selective and selective), with and without inhibitors (enzyme inhibitor or antagonists), to analyze the sensitivity of β-adrenoceptor subtypes as well as the expression of proteins associated with adipocyte lipolysis.

Methods

Animals

Male 10-week-old Wistar rats (HanUnib:WH, Rattus norvegicus, provided by Multidisciplinary Center for Biological Research - CEMIB, www.cemib.unicamp.br), 359.6 $\pm$ 6.2 g (n=26) were randomly divided into 2 groups: age-matched control (W) and L-NAME-induced hypertension (L-NAME, 40 mg/Kg/day). L-NAME administration was adapted from Paulis et al., 2010 (Paulis *et al.*, 2010). L-NAME was given in tap water, *per os*, for 5 weeks and its concentration was adjusted to water consumption and weight 3 days a week. The rats were housed in collective cages (3 rats per cage) at 22^o C on a 12 h light-dark cycle, with lights on at 06:30 a.m. All animals were given chow and water *ad libitum*. Control and treated rats were fasted for 12-16 h before sacrifice. The rats were anaesthetized with Zoletil 50® (tiletamine, 29 mg/kg and zolazepam, 29 mg/kg, i.m.; Vibac Laboratories, Carros, France) and Anasedan® (xylazine, 12,88 mg/kg, i.m.; Sespo Ind. e Com. Ltda, Paulínia, SP, Brazil) .The experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the Institute of Biology (UNICAMP, 2616-1).

Chemicals

Bovine serum albumin (fraction V), collagenase (type II), HEPES, (−)-isoproterenol hydrochloride, DL-propranolol hydrochloride, (+-)-metoprolol -(+)tartrate, caffeine, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20 and glycerol were from Sigma-Aldrich (St Louis, MO, USA). ICI 118,551 was obtained from Tocris Cookson, Inc. (Ballwin, MO). Salbutamol sulphate was obtained from Glaxo Smith Kline (London, UK). Kits for glycerol quantification were obtained from Laborclin (Pinhais, PR / Brazil). Nitrocellulose membrane (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Anti-β_1-AR (sc-568, rabbit polyclonal), anti-β_2-AR (sc-569, rabbit polyclonal), anti-β_3-AR (sc-1473, goat polyclonal), anti-GR (sc-56851, mouse monoclonal), anti-adenosine A1R (sc-7500, goat polyclonal), anti-adenosine A2R (sc-7504, goat polyclonal), anti-PDE3b (sc-20793, rabbit polyclonal), anti-ABHD5 (CGI-58, sc-102285, goat polyclonal), anti-ATGL (sc-67355, rabbit polyclonal) and anti-HSL (sc-25843, rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Gs (goat polyclonal, ab101736), anti-Gi (rabbit monoclonal, ab140125), and anti-β-actin (rabbit polyclonal, ab8227) were from Abcam (Cambridge, MA, USA). Anti-perilipin (#34675, rabbit polyclonal) was from Cell Signaling (Danvers, MA, USA).

Measurement of blood pressure

Arterial blood pressure was measured in 15-week-old anesthetized rats, according Conceição-Vertamatti et al. (Conceição-Vertamatti *et al.*, 2015), using a cannula introduced in the right carotid artery and attached with an ADI blood pressure module (MLS 370/7, ADInstruments - Australia). The data were acquired by Power Lab 8/30 data acquisition system and analyzed by Software LabChart Pro (ADInstruments – Australia). A separate group of animals was used only for this test. This group lived in the same vented chamber as the other rats, at the same time.

Adipocyte isolation and measurement of lipolysis

Adipocyte isolation was adapted from Crege *et al*., 2014 (Crege *et al.*, 2014). 2-3 g of epididymal fat depot was fragmented and digested in 50 mL polyethylene vials with 6 mL of Krebs-Ringer bicarbonate buffer, 25 mM Hepes, 6 mM glucose, and 3% bovine albumin (BSA fraction V fatty-acid free) pH 7.4 (KRBA buffer), with 1mg/mL collagenase (type II,

from *Clostridium histoliticum*), at 37°C with shaking (60 cycles/min) for 45 min. The cellular suspension was filtered through a nylon mesh and washed 3 times with 6 mL KRBA buffer (3% BSA). The final volume of cellular suspension was adjusted to 50 mL with KRBA buffer (3% BSA). 100 μL of cellular suspension were diluted in 900 μL of KRBA buffer and 10 μL of this suspension were transferred to a Mallassez chamber for adipocytes counting through light microscopy. Pharmacology assays were performed with 30,000 to 100,000 cells/mL that were incubated with shaking (60 cycles/min) in vials containing KRBA plus pharmacological agonists ((−)-isoproterenol hydrochloride 0.00001 μM to 10 μM (non-selective β-adrenoceptor agonist) or salbutamol 0.001 μM to 100 μM (selective β_2-adrenoceptor agonist) in a final volume of 1 mL for 60 min, with pre-incubation with antagonists or enzymatic inhibitor: DL-propranolol hydrochloride 1 μM (non-selective β_1 and β_2-adrenoceptor antagonist), (+-) - metoprolol -(+) tartrate 1 μM (selective β_1-adrenoceptor antagonist), ICI 118,551 hydrochloride 50 nM (selective β_2-adrenoceptor antagonist) or caffeine 100 μM (phosphodiesterase, PDE inhibitor and non-selective adenosine A1 and A2 receptor antagonist) for 60 min. At the end of incubation, the tubes were placed in an ice bath, after which their adipocytes were removed by aspiration. Samples of infranatant were used to glycerol determination, as an indicator of lipolysis. Basal lipolysis was determined in vials containing the cellular suspension without agonists. Glycerol quantification was done using kits from Laborclin (Pinhais, PR / Brazil), according manufacturer's instructions. Lipolytic response to agonists was calculated as follows: total glycerol value – basal glycerol value. All the results were expressed per million cells. Concentration-response curves for agonists were determined in the absence and presence of antagonists. All the results are expressed as μmol of glycerol. 10^6 cells during 60 min of incubation time at 60 cycles/min.

Western Blotting

Samples of epididymal adipose tissue (100-300 mg) were extracted from anesthetized rats and were immediately frozen in liquid nitrogen. Then they were stored in biofreezer (-80° C). They were thawed and homogenized in solubilization buffer at 4° C1% Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/mL] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Tissue homogenated was centrifuged for 40 min at 11,000 rpm in a 70.Ti rotor (Beckman) at 4°C to remove insoluble material. Bradford dye method was used to determine the protein

concentration of supernatants. Aliquots of the supernatants containing 0.025 mg of protein extracts were separated by SDSPAGE, transferred to nitrocellulose membranes and blotted with antibodies. Specific bands were detected by chemiluminescence and capture was performed with a Syngene GBox (Imgen Technologies, Alexandria, VA, USA). Additional information about each Western Blotting assay are presented in Supplementary figure 1.

Statistical analysis

The pharmachologycal data were evaluated as maximal response (Rmax,) to agonists. The values are shown as means $\pm$ s.e.m. The data normality was confirmed by Kolmogorov-Smirnov test and then we performed one-way Anova followed by Dunnet test or Student's t test to comparisons within the group (W or L-NAME) and Student's unpaired t test to comparisons between W and L-NAME. The quantification of protein expression was performed by densitometry analysis using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA), and the relative pixel intensity was normalized to β-actin. These values were further normalized to mean values of control group. All statistical analysis was done with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered significant for $p<0.05$.

Results

At the end of experiment, mean blood pressure was higher in L-NAME group than in W group (W: 106.9 $\pm$ 9.2 mm Hg versus L-NAME: 145.2 $\pm$ 12.4 mm Hg).

The responsiveness to β-adrenoceptor agonists was assessed in adipocytes isolated from epididymal depots of W and L-NAME rats. Basal glycerol release was not different between W and L-NAME groups (W: 0.96 ± 0.12 μmol glycerol. 10^6 cells/60 min *versus* L-NAME: 1.2 ± 0.17 μmol glycerol. 10^6 cells/60 min) No antagonist altered basal glycerol release, except caffeine, which increased it in L-NAME (2.4 ± 0.47 μmol of glycerol. 10^6 cells/60 min, $p<0.05$), but not in W group.

Maximal responses (Rmax) to isoproterenol were increased by ICI 118,551 (50 nM) in both groups (table 1, figure 1, $p<0.05$). Caffeine (100 μM) increased Rmax to salbutamol only in W group (table 1, figure 1c, $p<0.05$). Metoprolol (1 μM) and propranolol (1 μM) did not alter Rmax to isoproterenol of both groups (table 1, figure 1a and 1b). There were no differences between both groups in Rmax to isoproterenol. However, Rmax to salbutamol was

higher in L-NAME than in W (table 1, figure 1c and 1d, $p<0.05$). Propranolol increased the Rmax to isoproterenol in L-NAME rats compared with W (table 1, figure 1a and 1b, $p<0.05$).

Table 1: Maximal lipolytic responses (Rmax) of β-adrenoceptor agonists in the absence or presence of antagonists or enzyme inhibitor in adipocytes from L-NAME-induced hypertensive rats compared with control rats.

	W		L-NAME	
	Rmax	n	Rmax	n
Isoproterenol	2.091 ± 0.3517	12	2.420 ± 0.3684	8
Isoproterenol plus metoprolol (1μM)	1.994 ± 0.3354	12	2.636 ± 0.2817	8
Isoproterenol plus propranolol (1μM)	2.054 ± 0.2970	12	3.061 ± 0.2990$^{\#}$	8
Isoproterenol plus ICI 118,551 (50 nM)	3.259 ± 0.5758$^{@}$	12	3.676 ± 0.4490*	8
Salbutamol	2.496 ± 0.2826	5	3.427 ± 0.3299$^{\#}$	6
Salbutamol plus caffeine (100 μM)	5.032 ± 0.7057$^{@}$	5	4.630 ± 1.052	6

Maximal responses to the agonist were expressed in μmoL glycerol. 10^6cells/60min. $^{@}$ $P<0.05$ compared with the same group without antagonist, paired Student's t-test. * $P<0.05$ compared with the same group without antagonist, one-way Anova followed by Dunnet test. #$P<0.05$ compared with the W values, Student's unpaired t-test.

We also evaluated the expression of proteins of lipolytic pathway triggered by β-adrenergic activation and the adenosine receptors. We observed a decreased expression of β_1-AR (figure 2a), adenosine A2 receptor (adenosine A2R) (figure 2e) and perilipin (figure 2k) in L-NAME compared to W. L-NAME group also showed increased expression of HSL (figure 2n). We found no differences in the expression of β_2-AR (figure 2b), β_3-AR (figure 2c), adenosine A1R (figure 2d), glucocorticoid receptor (GR) (figure 2f), both stimulatory and inhibitory guanine nucleotide-binding protein (Gs and Gi respectively) (figures 2g and 2h), protein kinase A (PKA) (figure 2i), phosphodiesterase 3b (PDE3b) (figure 2j), adipose triacylglycerol lipase (ATGL) (figure 2m) and its co-activator, CGI-58 (comparative gene identification-58) (figure 2l).

Discussion

In our experimental conditions hypertension did not induces any alteration in basal lipolysis, which corroborates with CGI-58 and ATGL expression, that were the same in both groups. However, caffeine increases the basal glycerol release in L-NAME group, but not in W. It acts through antagonism of adenosine receptors of adipocytes, adenosine A1 receptor (associated to Gi proteins) and adenosine A2 receptor (associated to Gs proteins), and through

the inhibition of phosphodiesterase that hydrolyzes AMPc (Panchal *et al.*, 2012). Both antagonism of adenosine A1 receptor and inhibition of phosphodiesterase are involved with enhancement of hormone-induced lipolysis by caffeine (Panchal *et al.*, 2012), but it is able to stimulate lipolysis even in the absence of stimulatory ligands through antagonism of adenosine A1 receptor (Honnor, Dhillon e Londos, 1985), which is involved in basal lipolysis. This action was more potent in L-NAME probably because of two phenomena displayed by it: low expression of perilipins, which serves as a barrier to lipases in basal conditions, thus increasing basal lipolysis when little expressed (Sztalryd e Kimmel, 2014); low expression of adenosine A2 receptors, which is a Gs-coupled receptor, stimulating lipolysis following adenosine activation (Panchal *et al.*, 2012), whose antagonism prevents lipolysis less intensely in L-NAME.

There were no differences in Rmax to isoproterenol without antagonists between W and L-NAME. However, we observed alterations in the expression of two proteins related to stimulated lipolysis: increased HSL and decreased perilipin and adenosine A2 receptor in L-NAME group, which can raise (Large *et al.*, 1998) and diminish stimulated lipolysis respectively (Panchal *et al.*, 2012; Sztalryd e Kimmel, 2014). Therefore we suggest that alterations in HSL, perilipin and adenosine A2 receptor expression may offset each other, with no changes in lipolysis pathway triggered by a non-selective agonist in L-NAME group.

The increased Rmax in W and L-NAME in the presence of selective antagonism of β_2-AR (triggered by ICI 118,551) suggests that β_2-AR associates more with Gi protein than the classical Gs protein in both groups. This result differs from our previous studies (Farias-Silva *et al.*, 1999; Grassi-Kassisse *et al.*, 2003), in which ICI 118,551 didn't cause any effect on the lipolytic response of isolated adipocytes from control rats. The apparent paradox can be explained by different isoproterenol agonists used in the present study and in the previous ones: (−)-isoproterenol hydrochloride and (±) isoproterenol hydrochloride respectively. (−)-Isoproterenol hydrochloride induces more β-ARs phosphorylation than (±) isoproterenol hydrochloride (Sibley *et al.*, 1985), which raises PKA-mediated phosphorylation of the β_2-AR and consequently decreases its ability to couple to Gs, while dramatically increases its ability to couple to Gi (Xiao, 2001; Zamah *et al.*, 2002; Hill e Baker, 2003). Thus the ICI 118,551 antagonist releases inhibitory effect of β_2-AR by Gi.

L-NAME presented higher Rmax to salbutamol than W, which suggests increased β_2-AR mediated lipolysis, although its expression has remained unchanged between the groups. The up-regulation of β_2-AR function corroborates increased Rmax to isoproterenol in the

presence of propranolol in L-NAME compared to W, indicating a more important role of β_2-AR in inhibiting lipolysis than β_1-AR in stimulating it, which is also supported by low β_1-AR expression of L-NAME. Besides, there were no differences in Gs and Gi expression: so we suggest mechanisms other than protein expression, which can be implicated in increased β_2-AR function: high hormone affinity to β_2-AR and post-receptor mechanisms like increased function of stimulatory guanine nucleotide-binding protein.

Caffeine potentiates lipolytic response to salbutamol in W, but not in L-NAME. As discussed above, it enhances the availability and production of AMPc, and acts in hormone-induced lipolysis through inhibiting phosphodiesterase and antagonizing adenosine A1 and A2 receptors (Panchal *et al.*, 2012). It was not able to potentiate lipolytic response to salbutamol in L-NAME probably because of the potent action of this agonist on up-regulated β_2-AR in L-NAME adipocytes.

In summary, our results suggest alterations in β-adrenoceptor subpopulations concerning their function and expression as well as changes of lipolysis-associated proteins in L-NAME-induced hypertension. Increased β_2-AR function in hypertension supports our previous studies with acute stress models (swimming stress and footshock stress), in which stressed rats exhibited supersensitivity to isoproterenol, epinephrine and TA2005 (potent β_2-selective agonists) in both epididymal adipocytes (Farias-Silva *et al.*, 1999) and cardiac tissue (Marcondes *et al.*, 1996; Vanderlei *et al.*, 1996), which was abolished by ICI 118,551. We also observed down-regulation of β_1-AR, which corroborates our previous studies (Farias-Silva *et al.*, 2004). However, unlike one previous study with acute model of footshock stressed rats (Farias-Silva *et al.*, 2004), the current one didn`t display any differences in β_2-AR , β_3-AR and GR expression. Glucocorticoids are capable to cause these alterations, with stimulation of β_2-AR and inhibition of β_1-AR, β_3-AR and GR expression (Bakopanos e Silva, 2002). However L-NAME has presented higher levels of glucocorticoid than W (unpublished data), which may have altered β_1-AR expression, but not the other ones. We suggest that this heterogeneity comes from pathophysiologic factors associated with chronic stress and hypertensive status of the present hypertension model induced by L-NAME that differs from acute stress models. The β_2-AR up regulation can increase the importance of adrenaline as an endogenous stimulator of lipolysis (Grassi-Kassisse *et al.*, 2003), thus increasing catecholamine-induced lipolysis, which can cause metabolic disturbances associated with hypertension. Whether this effect occurs in hypertensive human and its relationship, if any, with metabolic disturbances in hypertension remain to be demonstrated.

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Figure legends

Figure 1: Dose-response curves to agonists with and without antagonists.
Isoproterenol in the absence (squares) or presence of antagonists metoprolol (circle), propranolol (upright triangle) and ICI 118,551 (inverted triangle) in adipocytes isolated from control rats (W) (A) (empty symbols) or hypertensive rats (L-NAME) (B) (full symbols). Salbutamol in the absence (x symbol) or presence of caffeine (asterisk) in adipocytes isolated from control rats (W) (C) or hypertensive rats (L-NAME) (D) The points are the means ± s.e.m. of 5-12 experiments performed in triplicate.

Figure 2: Western Blot analysis showing lipolysis-related protein expression of epididymal adipose tissue from control (W) and hypertensive (L-NAME) rats. Protein expression was normalized to β-actin, which was normalized to mean values of W. Cropped blots were used. Full-length blots are presented in Supplementary Fig 1.
Data are mean ± s.e.m. (W, n = 4; L-NAME, n=4-5). *, $p<0.05$.

Figure 1

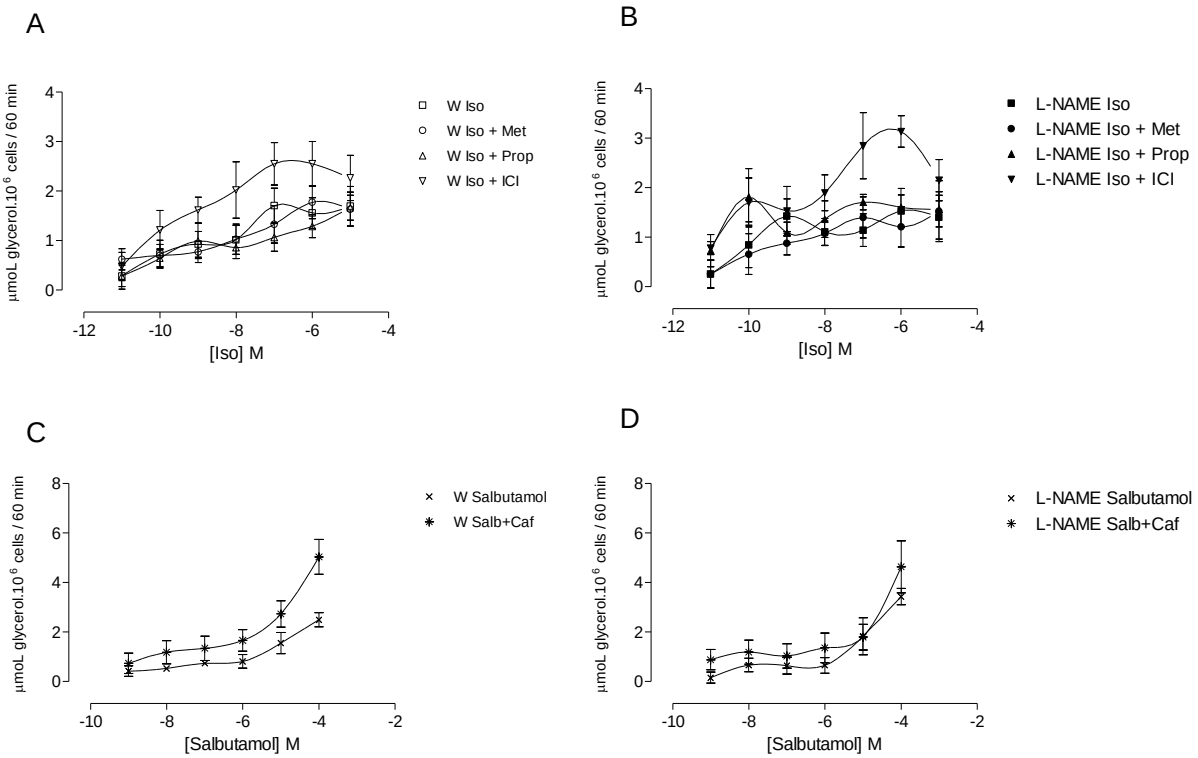
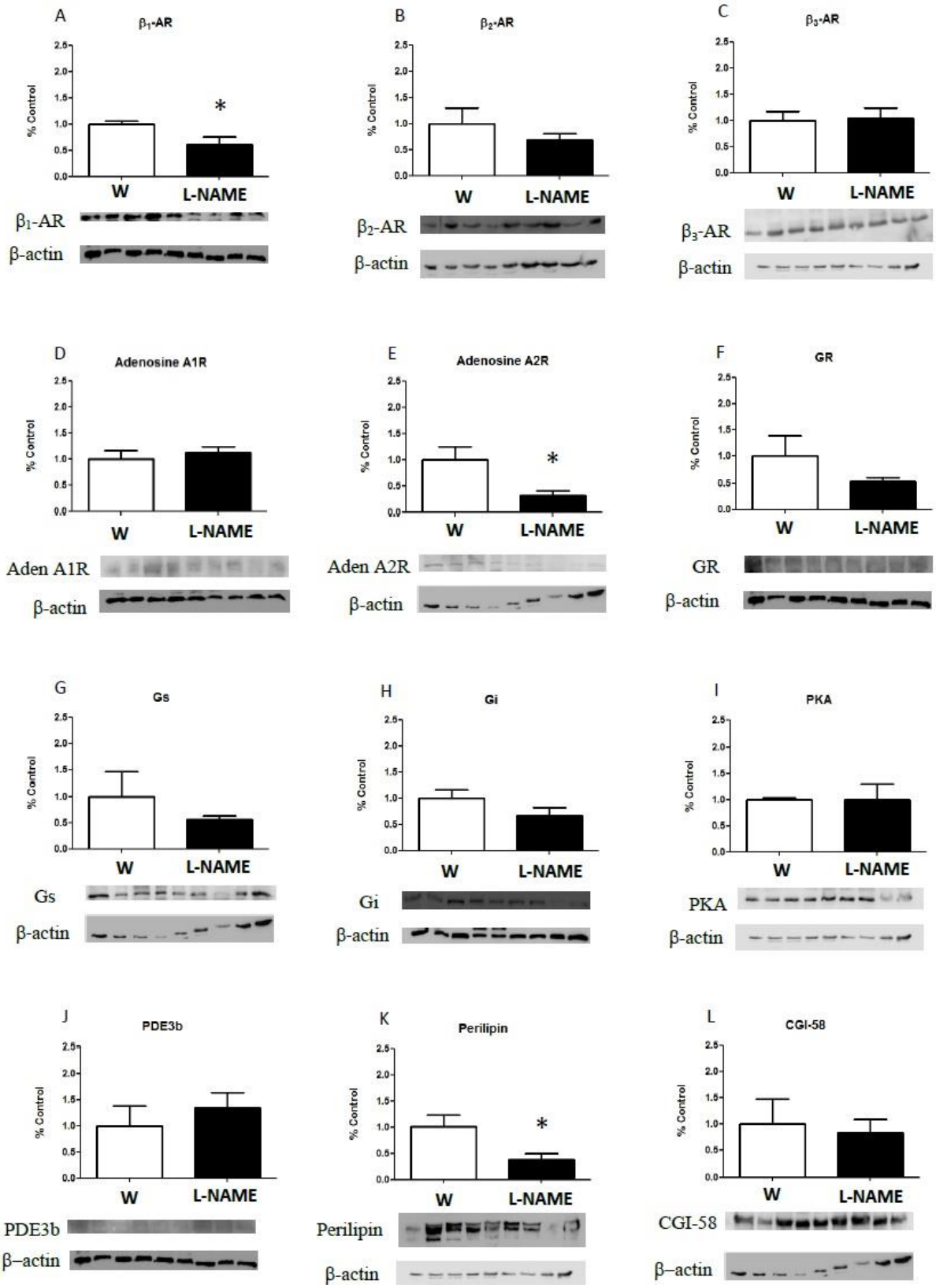
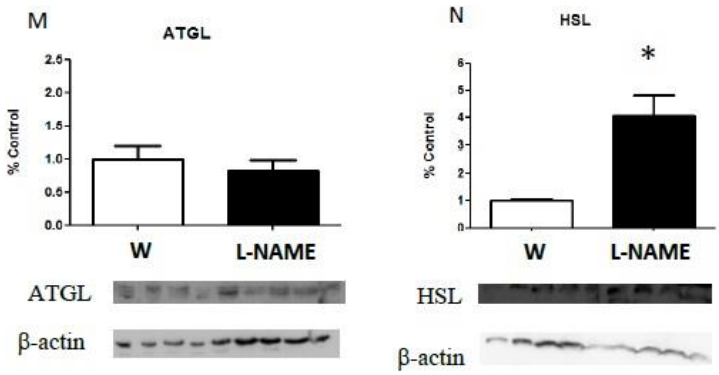

A
µmoL glycerol.10^6 cells / 60 min
[Iso] M
W Iso
W Iso + Met
W Iso + Prop
W Iso + ICI
B
µmoL glycerol.10^6 cells / 60 min
[Iso] M
L-NAME Iso
L-NAME Iso + Met
L-NAME Iso + Prop
L-NAME Iso + ICI
C
µmoL glycerol.10^6 cells / 60 min
[Salbutamol] M
W Salbutamol
W Salb+Caf
D
µmoL glycerol.10^6 cells / 60 min
[Salbutamol] M
L-NAME Salbutamol
L-NAME Salb+Caf

Figure 2: Western Blot analysis showing lipolysis-related protein expression of epididymal adipose tissue from control and hypertensive rats.

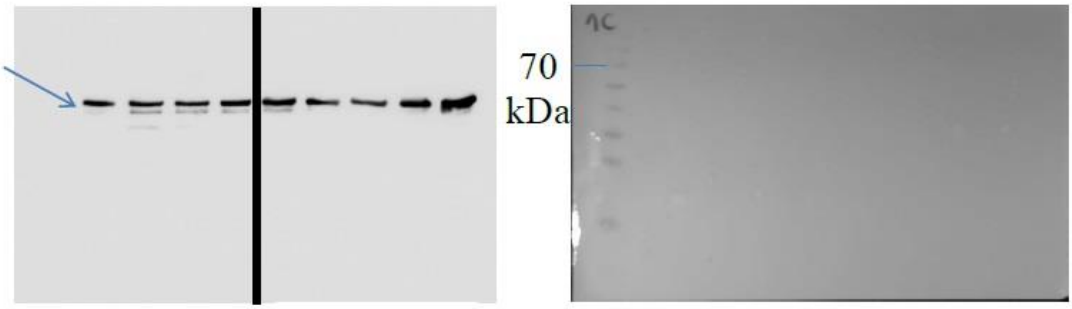
Data are mean ± s.e.m. (n = 4-5). *, $p<0.05$.



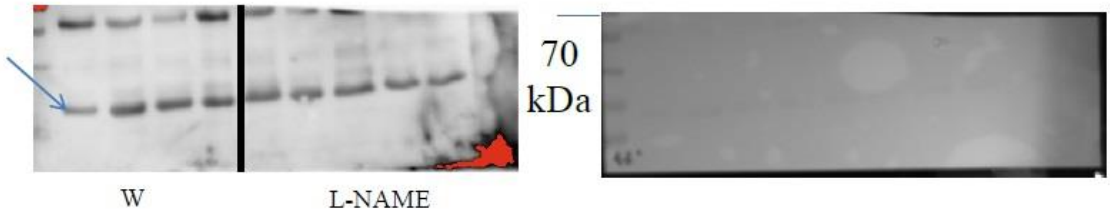

M
ATGL
% Control
2.5
2.0
1.5
1.0
0.5
0.0
W
L-NAME
ATGL
β-actin
N
HSL
*
% Control
6
5
4
3
2
1
0
W
L-NAME
HSL
β-actin

Supplementary figure 1. Original gel images of Western Blot for Figure 2 in the main text.

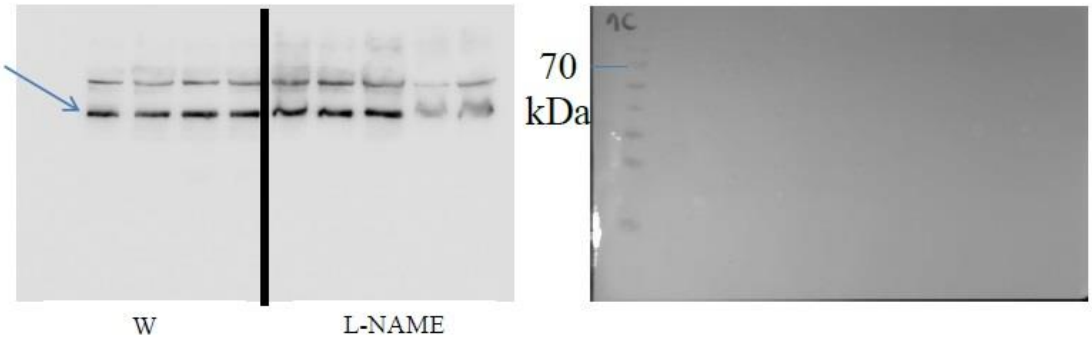
A) β-actin used to normalize β_3-AR, PKA and Perilipin. 2 strippings were performed before the blotting with β-actin.



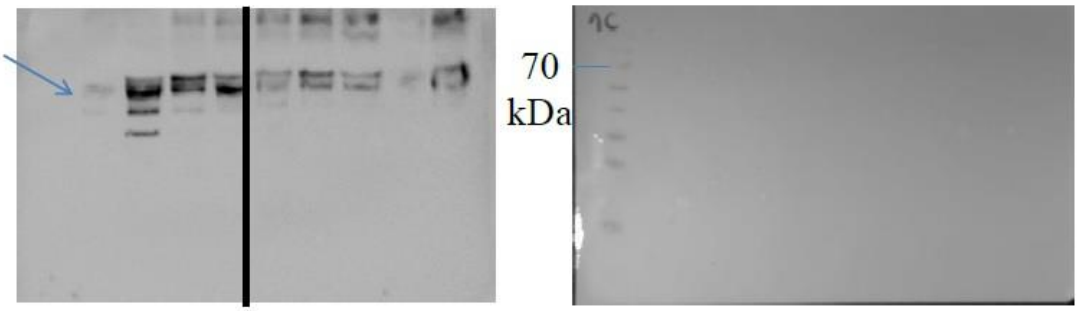
B) β_3-AR, no stripping was performed before the blotting with β_3-AR. β_3-AR and β-actin were from different membranes, containing the same samples.



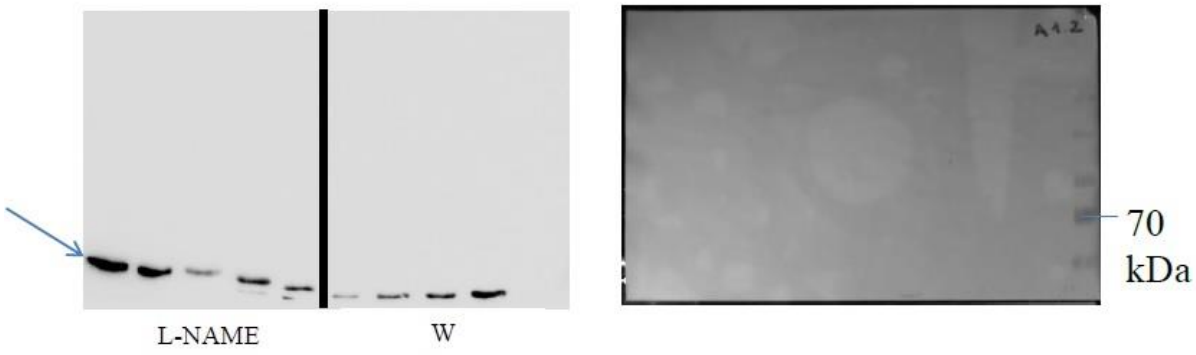
C) PKA, 8 strippings were performed before the blotting with PKA. PKA and β-actin were from the same membrane.



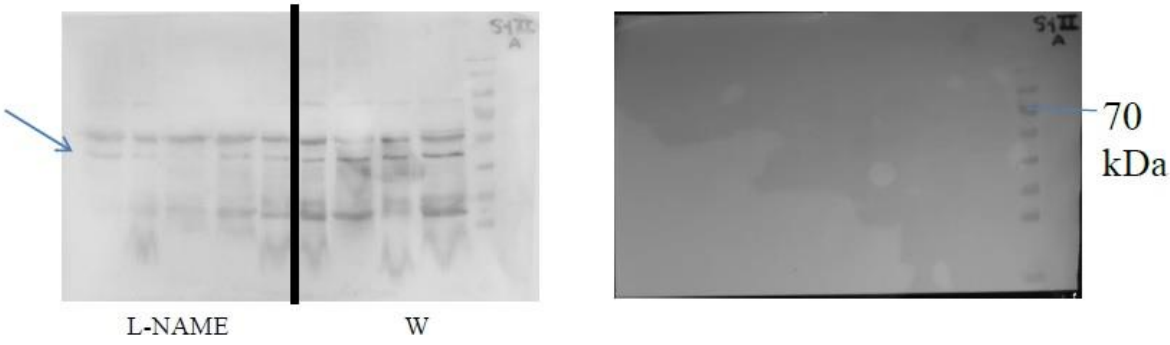
D) Perilipin, no stripping was performed before the blotting with Perilipin. Perilipin and β-actin were from the same membrane.



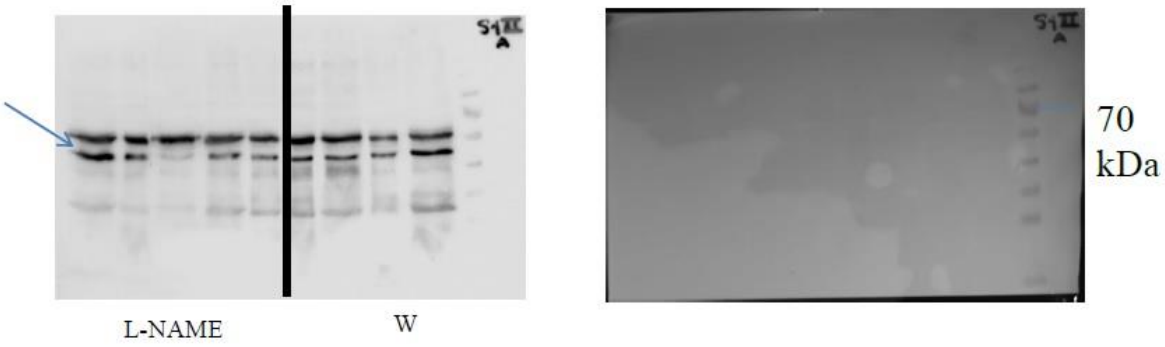
E) β-actin used to normalize adenosine A2 receptor, Gs and CGI-58. 2 strippings were performed before the blotting with β-actin.



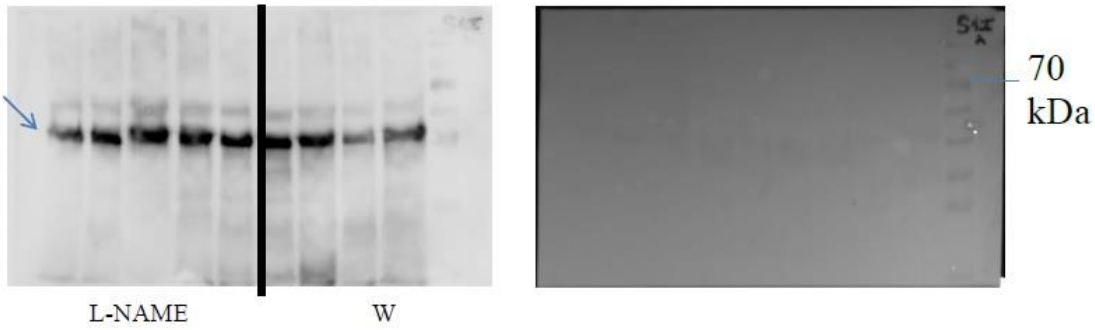
F) Adenosine A2R, 1 stripping was performed before the blotting with adenosine A2R. Adenosine A2R and β-actin were from different membranes, containing the same samples.



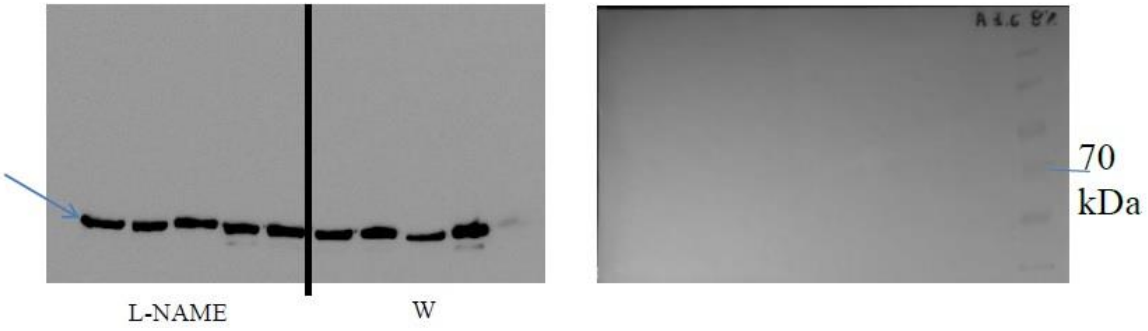
G) Gs, no stripping was performed before the blotting with Gs. Gs and β-actin were from different membranes, containing the same samples.



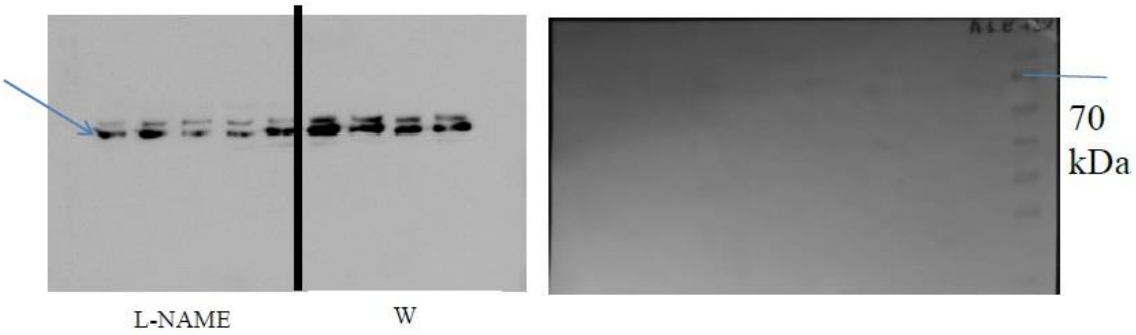
H) CGI-58, 1 stripping was performed before the blotting with CGI-58. CGI-58 and β-actin were from different membranes, containing the same samples.



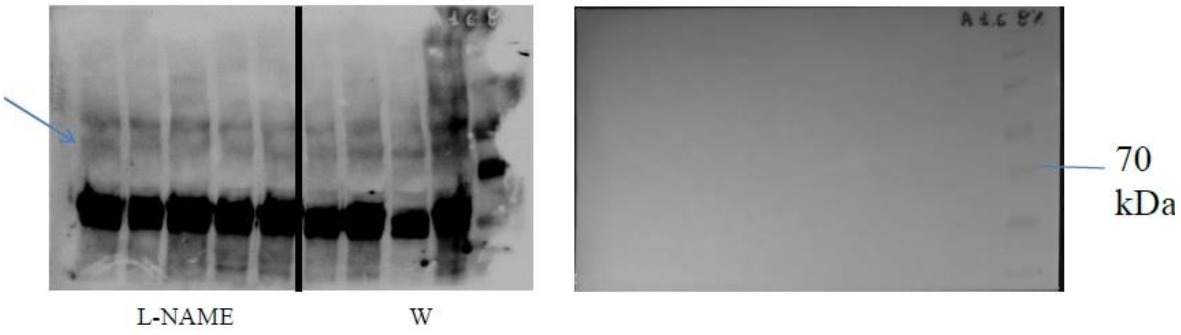
I) β-actin used to normalize β_1-AR, GR and PDE3b. No stripping was performed before the blotting with β-actin.



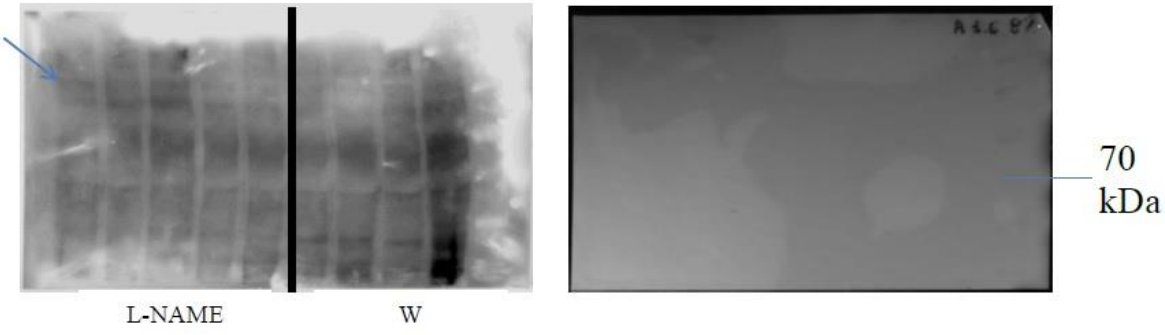
J) β_1-AR, 1 stripping was performed before the blotting with β_1-AR. β_1-AR and β-actin were from different membranes, containing the same samples.



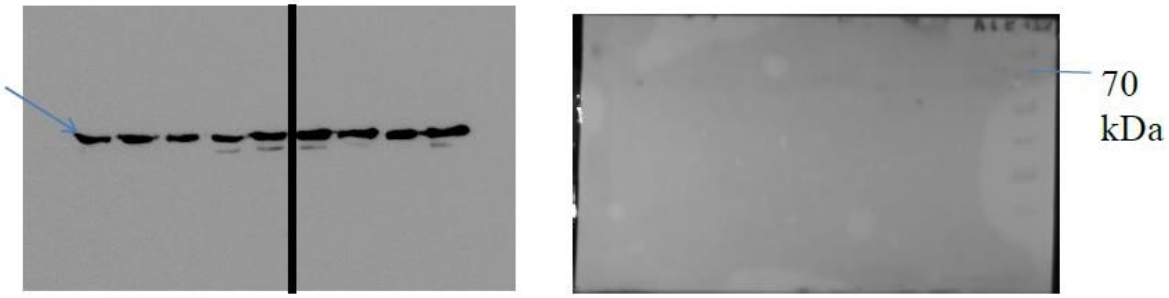
K) GR, 1 stripping was performed before the blotting with GR. GR and β-actin were from the same membranes.



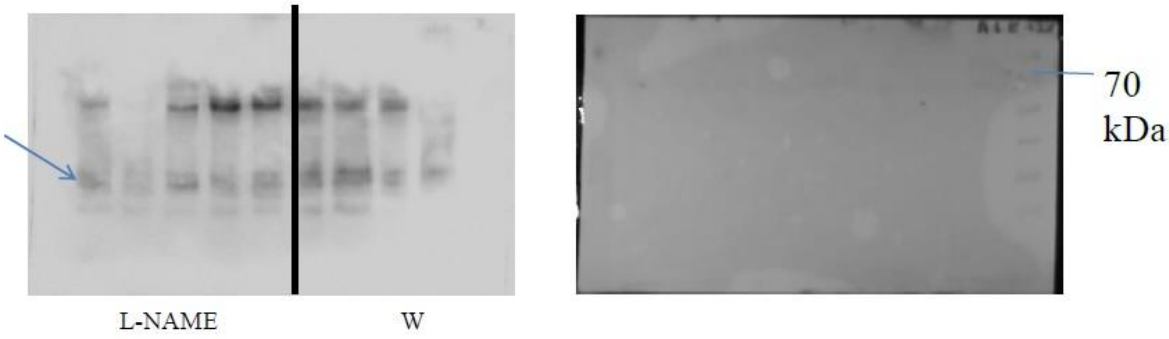
L) PDE3b, 3 strippings were performed before the blotting with PDE3b. PDE3b and β-actin were from the same membrane.



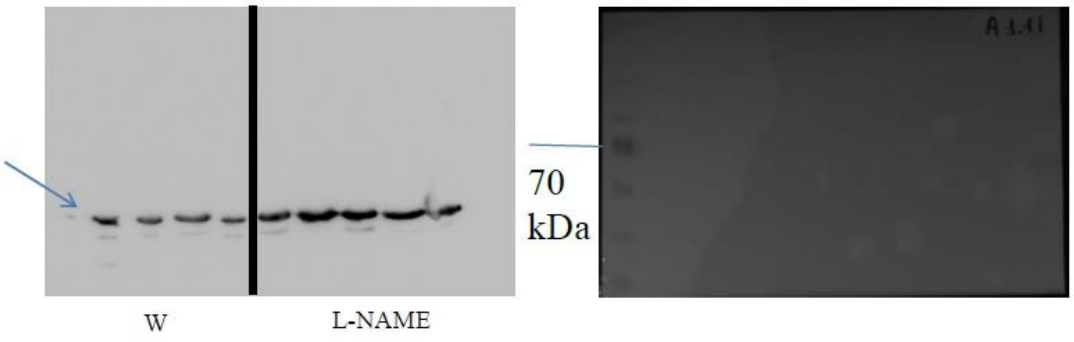
M) β-actin used to normalize Adenosine A1 receptor. No stripping was performed before the blotting with β-actin.



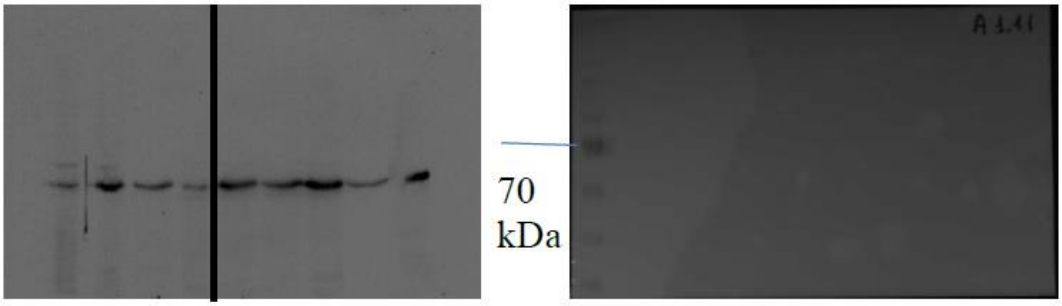
N) Adenosine A1 receptor, 5 strippings were performed before the blotting with Adenosine A1 receptor. Adenosine A1 receptor and β-actin were from the same membrane.



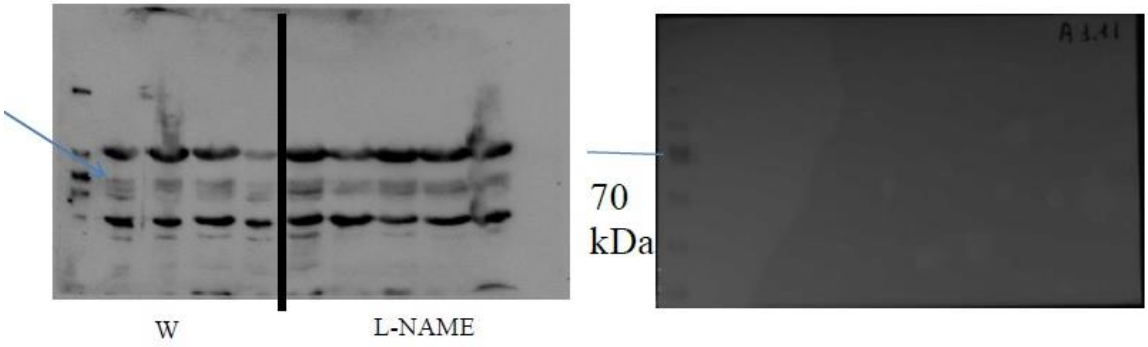
O) β-actin used to normalize β_2-AR and ATGL. 1 stripping was performed before the blotting with β-actin.



P) β_2-AR, no stripping was performed before the blotting with β_2-AR. β_2-AR and β–actin were from the same membrane.

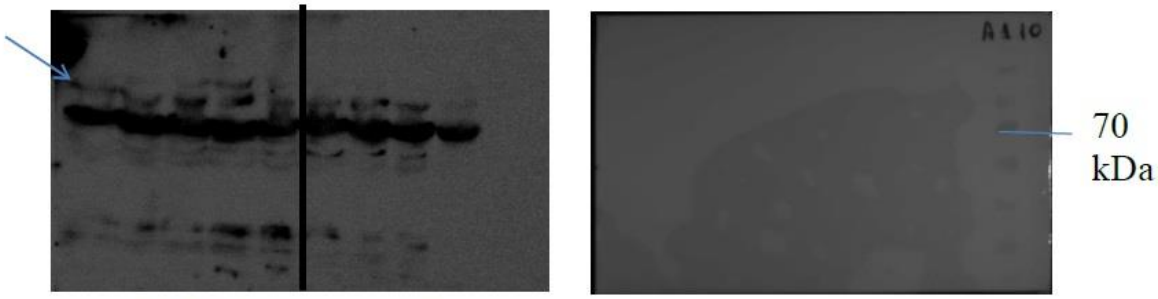


Q) ATGL, 3 strippings were performed before the blotting with ATGL. ATGL and β-actin were from the same membrane.

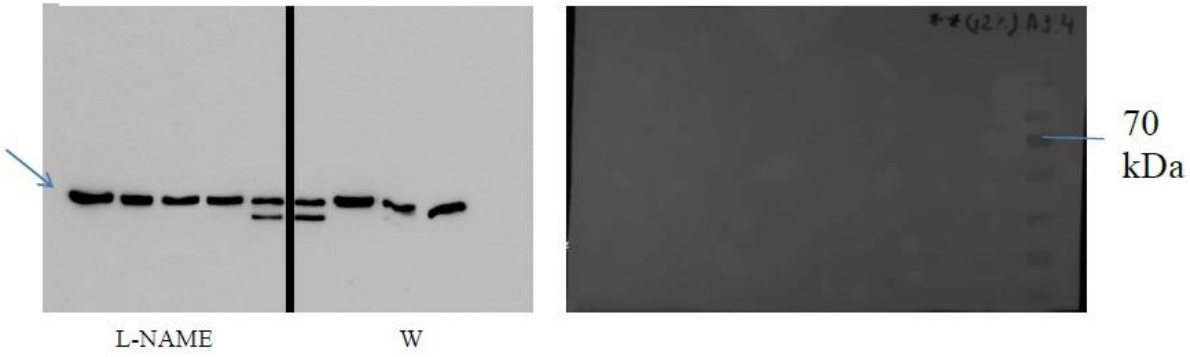


R) β-actin used to normalize HSL. 6 strippings were performed before the blotting with β-actin.

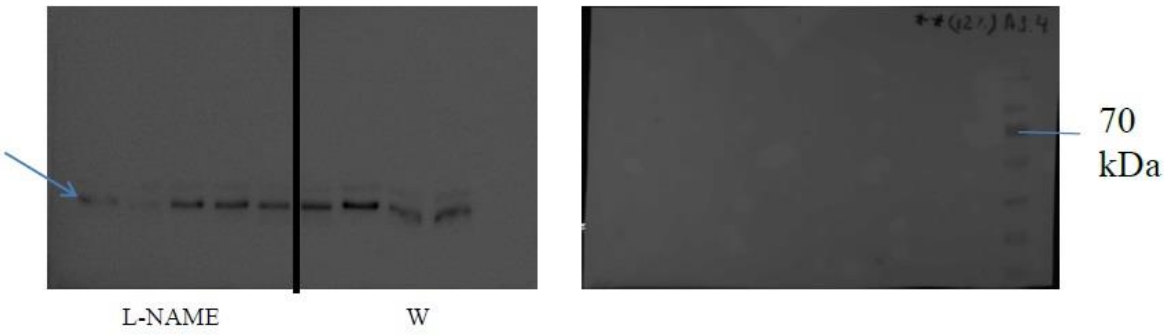
S) HSL, 5 stripping s were performed before the blotting with HSL. HSL and β–actin were from the same membrane.



T) β-actin used to normalize Gi. No stripping was performed before the blotting with β-actin.



U) Gi, 3 strippings were performed before the blotting with Gi. Gi and β-actin were from the same membrane.



β_2-Adrenoceptor Association to Gi in Epididymal Adipocytes from Spontaneously Hypertensive Rats

Running title: β_2-Adrenoceptor association to Gi in Hypertension

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Conflict of Interest Statement

All authors don`t have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Abstract

Many metabolic disturbances are associated with chronic sympathoexcitation, which is present in hypertension, a poorly clarified condition concerning metabolic alterations. Some studies with human and spontaneously hypertensive rats have observed decreased lipolysis following stimulation with noradrenaline and isoproterenol, without further molecular elucidation. This paper aims to investigate the lipolytic sensitivity of β-adrenoceptors subpopulations in isolated adipocytes and their expression in epididymal fat pads from hypertensive rats, associated with other proteins of lipolysis, and the involvement of adenosine. Male 15-week-old normotensive Wistar Kyoto (n=13) and Spontaneously Hypertensive Rats (n=15) were used in the experiments. Samples of epididymal adipose tissue were extracted for two assays: adipocytes isolation following pharmacological assays with non-selective (isoproterenol) and selective (salbutamol) β-agonists with and without antagonists (metoprolol 1 μM, propranolol 1 μM, ICI 118,551 50 nM and caffeine 100 μM); and Western Blot assays with epididymal fat depot. We observed increased maximal response to isoproterenol and its area under the curve in the presence of β_2-antagonist, ICI 118,551 in hypertensive rats, but not in normotensive ones. It suggested that β_2-adrenoceptor associated more to Gi than the classical Gs proteins, which was further supported by increased minimal and maximal responses to salbutamol and its area under the curve in the presence of caffeine in hypertensive rats. However we found no alterations in protein expression associated to this phenomenon. SHR epididymal fat pads presented increased perilipin and ATGL and these molecular alterations did not alter functional responses evaluated. Our major finding is a non-canonical pathway triggered by Gi-coupled-β_2-adrenoceptor activation in white adipocytes from spontaneously hypertensive rats, well described in cardio protective function of rodents. We suggest that Gi-coupled-β_2-adrenoceptor inhibition of adenylate cyclase can be involved in blunted lipolysis described in hypertensive human and SHR rats. However the regulation process, the circumstances under which this coupling may occur in white fat cells, along with its physiological consequences remain to be determined.

Introduction

Chronic stress may be implicated in many cardiac and metabolic disturbances, like hypertension, dyslipidemia, insulin resistance, visceral adiposity and obesity (Thorp e Schlaich, 2015), which together compose the metabolic syndrome (MetS) (Kaur, 2014).

Stress response is composed by two major arms: the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS), that are triggered by stressor stimuli to restore allostasis (Mcewen e Gianaros, 2011). According to many studies in human and animal models, chronic sympathoexcitation has a pivotal role in MetS (Thorp e Schlaich, 2015) and its conditions (Brooks *et al.*, 2015; Hering e Schlaich, 2015).

The effects of SNS activation in cardiovascular function is well known, through α- (arteriolar resistance) and β-adrenergic receptors (cardiac output); however its role in energy metabolism has been underexploited (Thorp e Schlaich, 2015).

Our group has investigated the effects of acute stress in some animal models in rat metabolism, specially the lipolytic response of isolated white adipocytes to catecholamines and adrenergic agonists (Farias-Silva *et al.*, 1999; Farias-Silva *et al.*, 2002; Sampaio-Barros *et al.*, 2003; Farias-Silva *et al.*, 2004), which occurs through β_1-, β_2- and β_3- adrenergic receptors or adrenoceptors (ARs) (Lafontan, 2012).

The catecholamine-induced lipolysis is mainly triggered by β_1-AR and β_2-AR in human adipocytes and by β_1-AR and β_3-AR in rat adipocytes under normal conditions (Lafontan, 2012). Lipolysis can be modulated by different substances, as adenosine. Adenosine is generated from adenosine triphosphate (ATP), and acts through its Gi- and Gs-coupled receptors, that are adenosine A1 and A2, respectively (Koupenova e Ravid, 2013). Adipocytes have both types, but there are more adenosine A1 receptor than A2; so adenosine exerts an inhibitory effect on lipolysis (Panchal *et al.*, 2012; Frühbeck *et al.*, 2014). The β-ARs expression and function can be modulated by catecholamines and glucocorticoids, which can cause desensitization and sensitization of β-AR subpopulations (Lafontan, 2012). We observed raised basal lipolysis and increased β_2-AR and decreased β_1-AR lipolytic responses in isolated epididymal adipocytes of footshock (Farias-Silva *et al.*, 1999) and swimming stressed rats (Sampaio-Barros *et al.*, 2003; Farias-Silva *et al.*, 2004).

Hypertension is another underappreciated condition concerning its metabolic effects. It seems to cause metabolic disturbances mainly through chronic sympathoexcitation (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011). Studies have suggested down-regulation of β-adrenoceptors caused by long-term SNS activation, which

can diminish thermogenesis of skeletal muscle and brown adipose tissue in hypertensive subjects (Van Marken Lichtenbelt e Schrauwen, 2011), predisposing to obesity. In this context, hypertensive patients have difficulties in losing their body weight (Wassertheil-Smoller *et al.*, 1992; Lasser *et al.*, 1995; Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011) and decrease their blood pressure after losing their body weight (Rekleiti, 2014). Hypertensive subjects have higher risk for developing obesity than normotensive ones, according to the Framingham Heart Study (Kannel *et al.*, 1967). However the underlying molecular mechanism, if it really is the β-AR-down-regulation, and how it occurs remain to be determined.

Hypertension has also been associated with disturbances in adipose tissue lipolysis. Hypertensive patients (Townsend e Klein, 1997) displayed lower blood glycerol levels than normotensive ones, when they were stimulated with noradrenaline and in basal conditions, suggesting β-AR desensitization in adipose tissue. Studies with the spontaneously hypertensive rats (SHR) also observed decreased lipolytic response to noradrenaline (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988) and isoproterenol (Nelson, Shepherd e Spitzer, 1987) when compared to Wistar Kyoto (WKY) control. They suggested many mechanisms which can be associated with the blunted lipolytic response: altered function of guanine nucleotide-binding protein, defective regulation of lipolytic enzymes at the protein kinase hormone-sensitive lipase level, low hormone affinity to β-adrenoceptors (Nelson, Shepherd e Spitzer, 1987) and/or decreased β-adrenoceptor density in SHR membrane (Chiappe De Cingolani, 1988). So they lack of further molecular elucidation about the function and expression of β-AR subpopulations as well as other components of lipolysis pathway.

The genetic model of hypertension represented by SHR and its control WKY is important because it has a lot of similarities of its pathophysiology with essential hypertension in humans (Trippodo e Frohlich, 1981; Dornas e Silva, 2011). Besides, the SHR displays increased sympathetic tone caused by oxidative stress in rostral ventrolateral medulla (RVLM), which is a major source of excitatory input to sympathetic preganglionic nerves and is critically involved in blood pressure control (Dickinson, 2007; Kishi e Hirooka, 2013). Therefore it is a well representative model of hypertension concerning metabolic disturbances which can be attributed not only to its similarities with human hypertension but also to chronic sympathoexcitation causing β-ARs alterations in adipose tissue.

In the present study, we evaluated lipolytic sensitivity of β-AR subpopulations triggered by agonists with and without antagonists of isolated epididymal adipocytes, and their expression associated with other proteins of lipolysis pathway in epididymal fat pads from rats with spontaneous hypertension (SHR) compared to its control (WKY)

Methods

Animals

Male 15-week-old normotensive WKY (NTacUnib:WKY) rats (n=13) and hypertensive SHR (SHR/NTacUnib) rats (n=15) (Rattus norvegicus), were used for the experiments. The rats were housed in collective cages (3 rats per cage) at 22^{o}C on a 12 h light-dark cycle, with lights on at 06:30 a.m. All animals were given chow and water *ad libitum*. Rats were starved for 12-16 h before sacrifice. The rats were anaesthetized with Zoletil 50® (tiletamine, 29 mg/kg and zolazepam, 29 mg/kg, i.m.; Vibac Laboratories, Carros, France) and Anasedan® (xylazine, 12.88 mg/kg, i.m.; Sespo Ind. e Com. Ltda, Paulínia,SP, Brazil). Rats were euthanized by anesthetic overload. The experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the Institute of Biology (UNICAMP, 2616-1).

Chemicals

Bovine serum albumin (fraction V), collagenase (type II), HEPES, (−)-isoproterenol hydrochloride, DL-propranolol hydrochloride, (+-)-metoprolol -(+)tartrate, caffeine, phenylmethylsulfonyl fluoride (PMSF), aprotinin, dithiothreitol, Triton X-100, Tween 20 and glycerol were from Sigma-Aldrich (St Louis, MO, USA). ICI 118,551 was obtained from Tocris Cookson, Inc. (Ballwin, MO). Salbutamol sulphate was obtained from GlaxoSmithKline (London, UK). Kits for glycerol quantification were obtained from Laborclin (Pinhais, PR / Brazil). Nitrocellulose membrane (BA85, 0.2 μm) was from Amersham (Aylesbury, UK). Anti-β_1-AR (sc-568, rabbit polyclonal), anti-β_2-AR (sc-569, rabbit polyclonal), anti-β_3-AR (sc-1473, goat polyclonal), anti-GR (sc-56851, mouse monoclonal), anti-adenosine A1R (sc-7500, goat polyclonal), anti-adenosine A2R (sc-7504, goat polyclonal), anti-ABHD5 (CGI-58, sc-102285, goat polyclonal), anti-ATGL (sc-67355, rabbit polyclonal) and anti-HSL (sc-25843, rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Gs (goat polyclonal, ab101736), anti-Gi (rabbit

monoclonal, ab140125) and anti-β-actin (rabbit polyclonal, ab8227) were from Abcam (Cambridge, MA, USA). Anti-perilipin (#34675, rabbit polyclonal) was from Cell Signaling (Danvers, MA, USA).

Measurement of blood pressure

Measurements of arterial blood pressure were performed in 15-week-old anesthetized rats, according Conceição-Vertamatti et al. (Conceição-Vertamatti *et al.*, 2015). A catheter was inserted into the right carotid artery to record arterial blood pressure via ADI blood pressure module (MSL 370/7, ADInstruments, Australia). Pressure signals were acquired by Power Lab 8/30 and analyzed by LabChart Pro Software (ADInstruments- Australia). A separate group of animals was used only for this test. This group lived in the same vented chamber as the other rats, at the same time.

Adipocytes isolation and measurement of lipolysis

Isolation of adipocytes was adapted from Crege *et al*., 2014 (Crege *et al.*, 2014). 2-3 g of epididymal adipose tissue was fragmented and digested with 1 mg/mL collagenase (type II, from *Clostridium histoliticum*), in polyethylene tubes with 6 mL of Krebs-Ringer bicarbonate buffer containing Hepes (25 mM), glucose (6 mM), and bovine albumin (3%, BSA fraction V fatty-acid free), pH 7.4 (KRBA), at 37^{o}C with shaking (60 cycles/min) during 45 min. The isolated fat cells were filtered through a nylon mesh and washed 3 times with 6 mL KRBA buffer (3% BSA). The final volume of cellular suspension was adjusted to 50 mL with KRBA buffer (3% BSA). 100 μL of cellular suspension were adjusted with KRBA to a 10% suspension: 10 μL of this suspension were transferred to a Mallassez chamber for adipocytes counting through light microscopy. 30,000 to 100,000 cells were incubated with gentle shaking (60 cycles/min) in vials containing KRBA plus pharmacological agonists ((−)-isoproterenol hydrochloride 0.00001 μM to 10 μM (non-selective β-adrenoceptor agonist) or salbutamol 0.001 μM to 100 μM (selective β_2-adrenoceptor agonist) in a final volume of 1 mL for 60 min, with pre-incubation with antagonists: DL-propranolol hydrochloride 1 μM (non-selective β_1 and β_2-adrenoceptor antagonist), (+-) - metoprolol -(+) tartrate 1 μM (selective β_1-adrenoceptor antagonist), ICI 118,551 hydrochloride 50 nM (selective β_2-adrenoceptor antagonist) or caffeine 100 μM (phosphodiesterase, PDE inhibitor and non-selective adenosine A1 and A2 receptor antagonist) for 60 min. After incubation, the tubes were placed in an ice bath, after which their adipocytes were removed by aspiration. Samples

of infranatant were used to glycerol determination, as an indicator of lipolysis. Basal lipolysis was determined in vials containing the cellular suspension without agonists (with or without antagonists). Glycerol quantification was done using kits from Laborclin (Pinhais, PR / Brazil), according manufacturer's instructions. The lipolytic response to agonists was calculated by subtracting the basal glycerol amount of the total amount of glycerol observed in the suspension incubated with the agonist. All the results were expressed per million cells. Concentration-response curves for agonists were determined in the absence and presence of antagonists.

Western Blotting

Fragments of epididymal fat depot (100-300 mg) were isolated from anesthetized rats and were immediately frozen in liquid nitrogen, and then stored in biofreezer (-80^{o}C). They were thawed and homogenized in solubilization buffer at 4^{o}C1% Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/mL] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Homogenized issue was centrifuged for 40 min at 11,000 rpm in a 70.Ti rotor (Beckman) at 4^{o}C to remove insoluble material. Protein concentration of supernatants was obtained by Bradford. Aliquots of the supernatants containing 0.025 mg of protein extracts were separated by SDS PAGE, transferred to nitrocellulose membranes and blotted with antibodies. Specific bands were detected by chemiluminescence and capture was performed with a Syngene GBox (Imgen Technologies, Alexandria, VA, USA). Densitometry analysis by UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA) was used to quantify protein expression, whose pixel intensities were normalized to β-actin, which were normalized to mean values of WKY group. Details of Western Blotiing assay were showed in Supplementary figure 1.

Statistical analysis

The pharmachologycal data were evaluated as minimum and maximal response (Rmin and Rmax, respectively) and in total response, i.e. area under curve (AUC). The values are shown as means $\pm$ s.e.m. The normality was confirmed by Kolmogorov-Smirnov test and then one-way Anova followed by Dunnet test or Student's *t*-test were used for comparisons in the same group and unpaired Student's *t*-test was used for comparisons between WKY and SHR. All statistical analysis was done with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered significant for $p<0.05$.

Results

We performed functional assays with isolated epididymal adipocytes to evaluate their responsiveness of β-AR subpopulations. Basal glycerol release of adipocytes from SHR (0.89 ± 0.21 μmol glycerol. 10^6 cells/60 min) was not different from WKY (0.83 ± 0.20 μmol glycerol. 10^6 cells/60 min). Caffeine (100 μM) was able to increase basal glycerol release of both groups (WKY: 2.68 ± 0.45 μmol glycerol. 10^6 cells/60 min *versus* SHR: 2.14 ± 0.28 μmol glycerol. 10^6 cells/60 min, $p<0.05$). No alterations were observed in the presence of the other antagonists.

No antagonist was able to alter maximal (Rmax) and minimal (Rmin) responses to isoproterenol (table 1, figure 1a) and salbutamol (table 1, figure 1c) and the area under the curve (AUC) of isoproterenol of WKY (table 1, figure 1a); except the pretreatment with caffeine (PDE inhibitor and adenosine receptor antagonist) raised AUC of salbutamol in this group (table 1, figure 1c, $p<0.05$). However SHR displayed increased Rmax and AUC of isoproterenol in the presence of ICI 118,551 (50 nM) (table 1, figure 1b, $p<0.05$) and elevated Rmax, Rmin and AUC of salbutamol, in the presence of caffeine (table 1, figure 1d, $p<0.05$). Metoprolol (1 μM) and propranolol (1 μM) were not able to change any parameters of isoproterenol curves in SHR (table 1, figure 1b).

When we compared both groups, SHR showed higher values of AUC of isoproterenol in the presence of ICI 118,551 (table 1, figure 1a and 1b, $p<0.05$) and of Rmin to salbutamol in the presence and absence of caffeine (table 1, figure 1c and 1d, $p<0.05$) than WKY.

Table 1: Maximal and minimal lipolytic responses to β-adrenoceptor agonists and area under the curve in the absence or presence of β-adrenoceptor antagonists in adipocytes from control and genetic hypertensive rats

	WKY				SHR			
	Rmax	Rmin	AUC	n	Rmax	Rmin	AUC	n
Isoproterenol								
No antagonist	2.62 ± 0.5692	0.49 ± 0.17	8.78 ± 1.45	8	1.86 ± 0.34	0.54 ± 0.14	6.26 ± 1.17	9
Metoprolol (1μM)	2.20 ± 0.5142	0.37 ± 0.10	7.79 ± 1.66	7	2.06 ± 0.35	0.57 ± 0.21	6.99 ± 1.76	9
Propranolol (1μM)	2.53 ± 0.3939	0.88 ± 0.30	9.89 ± 2.13	7	1.83 ± 0.23	0.46 ± 0.11	6.75 ± 0.82	9
ICI 118,551 (50 nM)	2.31 ± 0.3933	0.32 ± 0.08	7.93 ± 1.57	8	2.50 ± 0.40*	0.43 ± 0.14	8.45 ± 1.65$^{@}$	9
Salbutamol								
No antagonist	2.05 ± 0.2024	0.19 ± 0.04	4.02 ± 0.58	5	1.96 ± 0.23	0.37 ± 0.08$^{\#}$	4.67 ± 0.33	6
Caffeine (100 μM)	3.44 ± 0.4672	0.55 ± 0.21	8.67 ± 1.74$^{@}$	5	4.65 ± 0.59$^{@}$	2.36 ± 0.45$^{@\#}$	16.72 ± 1.66$^{@\#}$	6

The values are the means ± s.e.m. of the number of experiments (n) done in triplicate. Values are expressed in μol of glycerol released by 10^6 cells during 60 min. $^{@}$ P<0.05 compared with the same group without antagonist, Student's paired t-test. * P<0.05 compared with the same group without antagonist, one-way Anova followed by Dunnet test. #P<0.05 compared with the WKY values, Student's unpaired t-test.

We also assessed protein expression of β-AR subpopulations and of other molecules of lipolysis pathway. We found increased expression of perilipin (figure 2j) and ATGL (figure 2l) in SHR when compared to WKY. However there were no differences in the expression of β_1-AR (figure 2a), β_2-AR (figure 2b), β_3-AR (figure 2c), adenosine A1 receptor (adenosine A1R) (figure 2d) and adenosine A2 receptor (adenosine A2R) (figure 2e), glucocorticoid receptor (GR) (figure 2f), both stimulatory (figure 2g) and inhibitory (figure 2h) guanine nucleotide-binding protein (Gs and Gi respectively), protein kinase A (PKA) (figure 2i), co-activator of ATGL, CGI-58 (comparative gene identification-58) (figure 2k) and hormone-sensitive lipase (HSL) (figure 2m).

Discussion

Basal glycerol release was not different between groups; however we observed alterations in the expression of two proteins related to basal lipolysis: increased ATGL and perilipin in SHR group, which can increase and decrease basal lipolysis respectively (Bézaire

e Langin, 2009; Sztalryd e Kimmel, 2014). So we suggest that both alterations in ATGL and perilipin expression may offset each other, with no changes in basal lipolysis. Besides, it corroborates with the unchanged CGI-58 expression, which is a co-factor that stimulates ATGL and is involved in basal lipolysis (Bézaire e Langin, 2009; Sztalryd e Kimmel, 2014).

Caffeine raised basal lipolysis of both groups. It acts on adipocytes through different mechanisms: caffeine inhibits phosphodiesterase, which hydrolyzes AMPc, originated by lipolysis activation (Panchal *et al.*, 2012), and it antagonizes adenosine receptor A1, a Gi-coupled receptor, which prevents lipolysis when stimulated by adenosine, through inhibition of adenylate cyclase (Panchal *et al.*, 2012; Yang *et al.*, 2015). Caffeine is also antagonist of adenosine A2 receptors, which are Gs-coupled receptors and promote lipolysis when activated by adenosine, but they are present at lower levels than adenosine A1 receptors in white adipocytes (Rines, Verdeguer e Puigserver, 2015). In the absence of any lipolytic hormones, like in the basal lipolysis, caffeine acts mainly through antagonism of adenosine A1 receptor, which is sufficient to produce a large increase in PKA activity, thus increasing lipolysis (Honnor, Dhillon e Londos, 1985). We suggest that the similar effect of caffeine on both groups were due to no differences in the expression of adenosine A1 and A2 receptors between WKY and SHR.

The selective β_2-AR antagonist (ICI 118, 551) increased Rmax to isoproterenol and its AUC in SHR, while it caused no effects on WKY. It suggests that this antagonist released β_2-AR from inhibition in SHR, which can be attributed to its association to Gi more than the classical Gs protein. It can be explained by the promiscuous role of β_2-AR in its association to G proteins: Gs under physiologic conditions and Gi at high levels of receptor expression (Hill e Baker, 2003) and under extreme conditions (Taylor, 1990), although we didn't find any differences concerning Gi and Gs expression between both groups.

However, our previous studies with acute footshock stressed rats (Farias-Silva *et al.*, 1999; Farias-Silva *et al.*, 2004) showed increased β_2-AR-mediated lipolysis and decreased β_1-AR lipolytic response, whereas β_3-AR seemed to be resistant to stress effects. These functional results were accompanied by augmented β_2-AR expression and diminished β_1- , β_3-AR and GR expression (Farias-Silva *et al.*, 2004). Such differences between the previous and the present studies can be attributed to glucocorticoids effects and the hypertensive status. Glucocorticoids stimulate β_2-AR expression while inhibits β_1-AR, β_3-AR (Bakopanos e Silva, 2002) and GR (Deak *et al.*, 1999), which probably altered β-subpopulations in footshock stressed rats. On the other hand, we found no differences in glucocorticoid levels between

WKY and SHR (unpublished data), which corroborates with β_1-, β_2-, β_3-AR and GR expression, that were the same in both groups. Besides, the hypertensive status can be an extreme condition that can trigger the protective role of the promiscuous β_2-AR association to Gi (Taylor, 1990), which opposes β_1-AR and β_3-AR effects on lipolysis and cardiac function (Xiao, 2001).

This phenomenon is further supported by the lipolytic responses of adipocytes to the selective β_2-agonist (salbutamol) with and without caffeine. Caffeine effects on salbutamol-mediated lipolysis were minimal in WKY: it was able to raise only AUC, but not Rmax and Rmin. However it increased Rmax and Rmin to salbutamol, as well as its AUC in SHR; besides Rmin and AUC of salbutamol in the presence of caffeine was higher in SHR than WKY. Caffeine raises hormone-stimulated lipolysis through inhibition of phosphodiesterase and through antagonism of adenosine A1 receptor (Panchal *et al.*, 2012), as discussed above. As no differences were found in both groups, concerning the expression of these proteins, we attributed the differential effect of caffeine to the alteration of β_2-AR in SHR. Thus, we suggest that the effect of caffeine on inhibition of phosphodiesterase was limited in WKY, because its β_2-AR participation on lipolysis is minimal under normal conditions (Lafontan, 2012). However caffeine was more potent in SHR probably due to its counteracting effects of β_2-AR association to Gi. Furthermore, increased perilipin expression in SHR was not sufficient to raise agonist-stimulated lipolysis probably due to the inhibitory effects of β_2-AR association to Gi.

In conclusion, our major finding is a non-canonical pathway triggered by Gi-coupled-β_2-AR activation in white epididymal adipocytes from Spontaneously Hypertensive Rats, which is well described in cardiac function of rodents (Xiao, 2001; Liu *et al.*, 2009; Fu e Xiang, 2015). This mechanism may have a protective role for cardiomyocytes, preventing the overstimulation of cardiac contraction, in the presence of high levels of catecholamines, triggered by stress response (Liu *et al.*, 2009; Fu e Xiang, 2015). It also stimulates cell survival in these cells through phosphoinositide 3-kinase (PI3K)-Akt (also known as protein kinase B) signaling pathway (Xiao, 2001; Fu e Xiang, 2015). We suggest that Gi-coupled-β_2-AR inhibition of adenylate cyclase can be involved in the blunted lipolysis described in hypertensive human (Townsend e Klein, 1997) and SHR rats (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988), and may also assume a protective role in fat, counteracting β_1- and β_3-AR mediated lipolysis, in the same manner as in cardiomyocytes, and adenosine is not involved with this phenomenon. However the

regulation process, the circumstances under which this coupling may occur in white fat cells, along with its physiological consequences remain to be determined.

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Conflict of Interest Statement

All authors don`t have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Figure Legends

Figure 1: Dose-response curves to agonists with and without antagonists.
Isoproterenol in the absence (squares) or presence of antagonists metoprolol (circle), propranolol (upright triangle) and ICI 118,551 (inverted triangle) in adipocytes isolated from control rats (WKY) (A) (empty symbols) or hypertensive rats (SHR) (B) (full symbols). Salbutamol in the absence (x symbol) or presence of caffeine (asterisk) in adipocytes isolated from control rats (WKY) (C) or hypertensive rats (SHR) (D) The points are the means ± s.e.m. of 5-9 experiments performed in triplicate.

Figure 2: Western Blot analysis showing lipolysis-related protein expression of epididymal adipose tissue from control (WKY) and hypertensive (SHR) rats. Protein expression was normalized to β-actin, which was normalized to mean values of WKY. Cropped blots were used. Full-length blots are presented in Supplementary Fig 1.
Data are mean ± s.e.m. (WKY, n = 4; SHR, n=4-5). *, $p<0.05$.

Figure 1

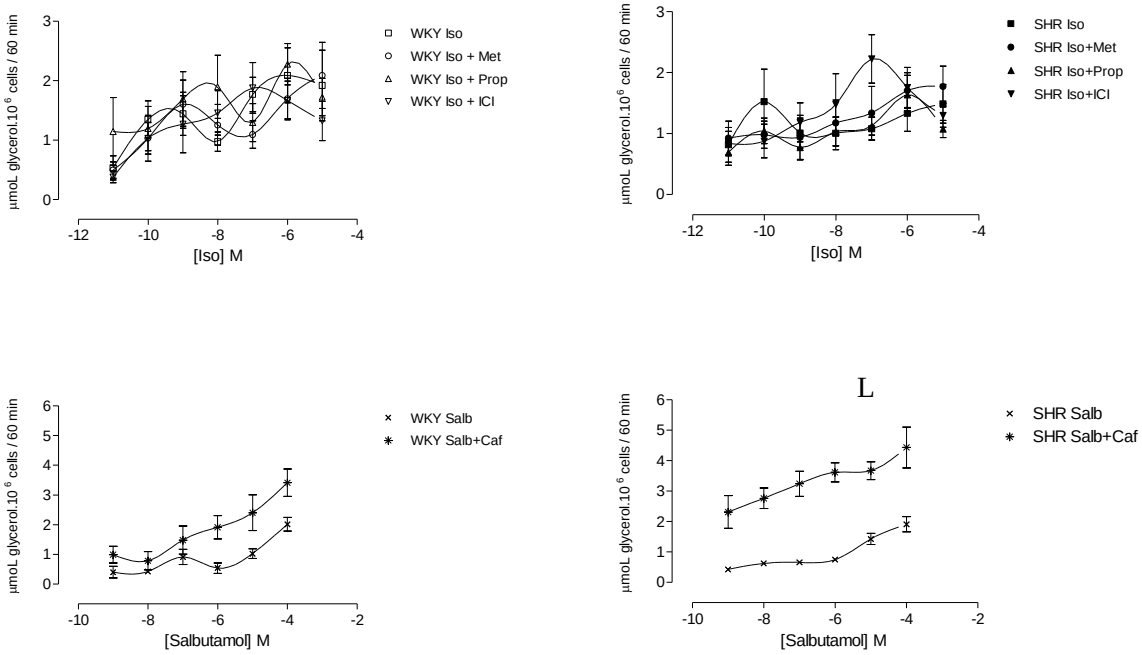
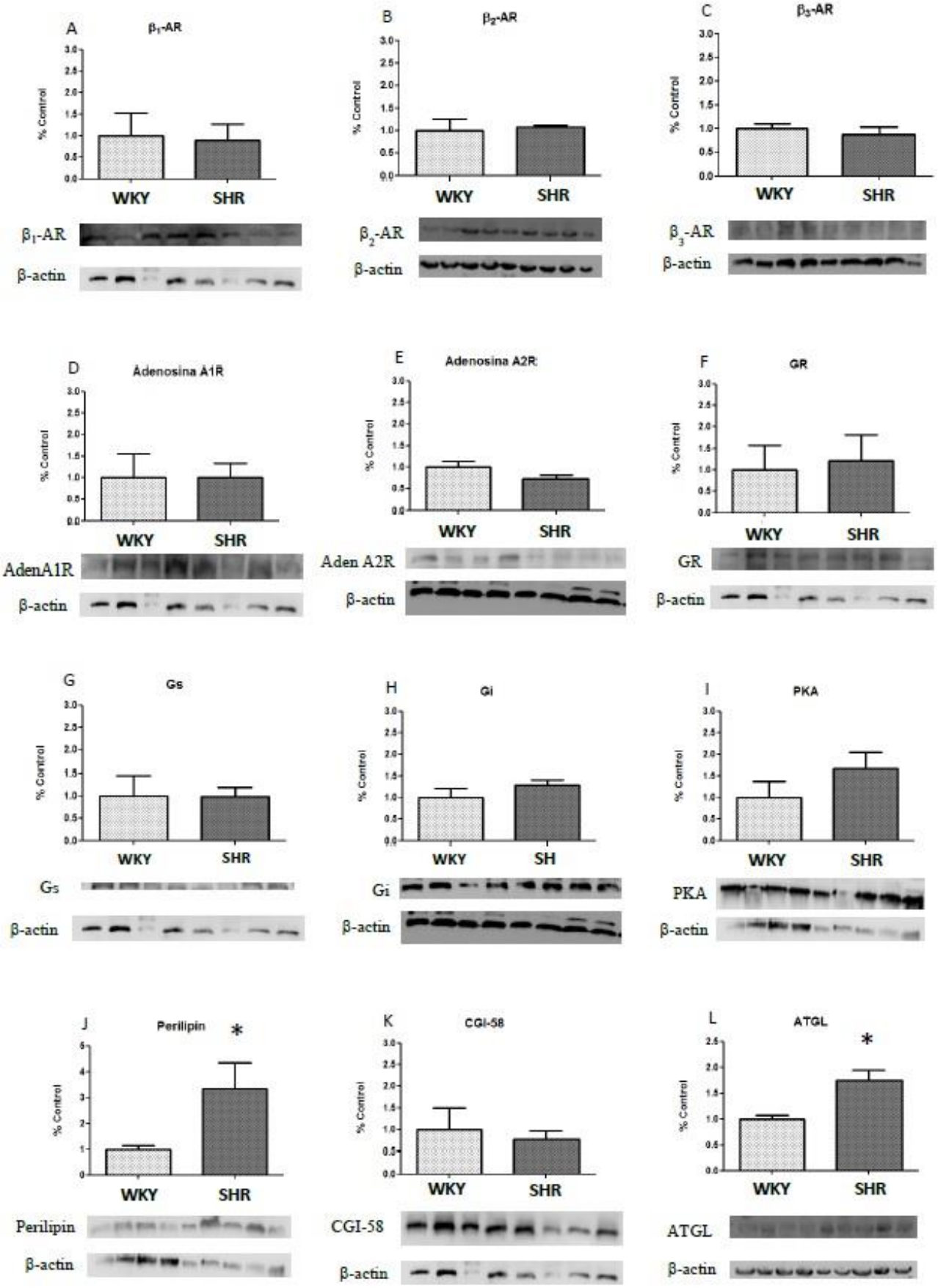
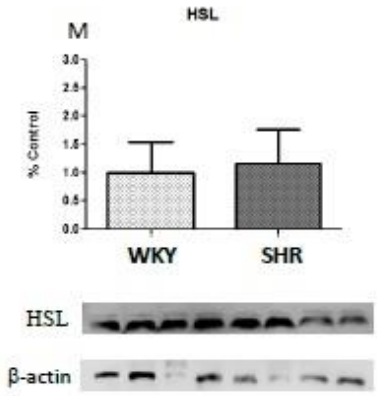

µmoL glycerol.10^6 cells / 60 min
[Iso] M
WKY Iso
WKY Iso + Met
WKY Iso + Prop
WKY Iso + ICI
SHR Iso
SHR Iso+Met
SHR Iso+Prop
SHR Iso+ICI
[Salbutamol] M
WKY Salb
WKY Salb+Caf
SHR Salb
SHR Salb+Caf
L

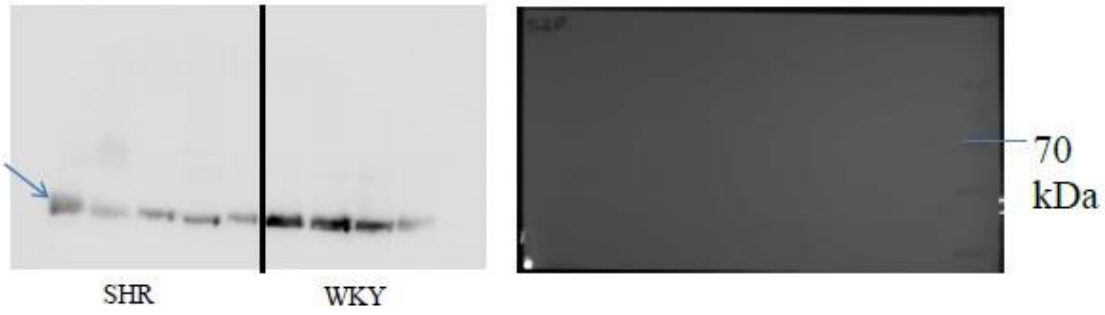
Figure 2


A
β1-AR
% Control
WKY
SHR
β1-AR
β-actin
B
β2-AR
% Control
WKY
SHR
β2-AR
β-actin
C
β3-AR
% Control
WKY
SHR
β3-AR
β-actin
D
Adenosina A1R
% Control
WKY
SHR
AdenA1R
β-actin
E
Adenosina A2R
% Control
WKY
SHR
Aden A2R
β-actin
F
GR
% Control
WKY
SHR
GR
β-actin
G
Gs
% Control
WKY
SHR
Gs
β-actin
H
Gi
% Control
WKY
SH
Gi
β-actin
I
PKA
% Control
WKY
SHR
PKA
β-actin
J
Perilipin
*
% Control
WKY
SHR
Perilipin
β-actin
K
CGI-58
% Control
WKY
SHR
CGI-58
β-actin
L
ATGL
*
% Control
WKY
SHR
ATGL
β-actin

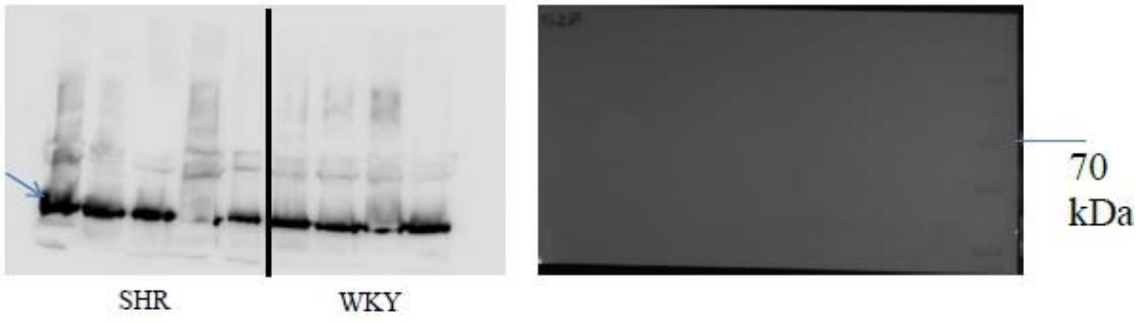

HSL
M
% Control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WKY
SHR
HSL
β-actin

Supplementary figure 1. Original gel images of Western Blot for Figure 2 in the main text.

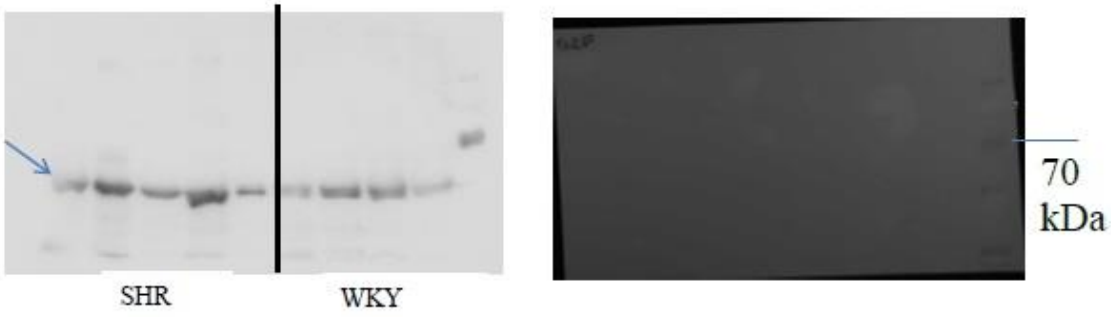
A) β-actin used to normalize PKA and Perilipin. 5 strippings were performed before the blotting with β-actin.



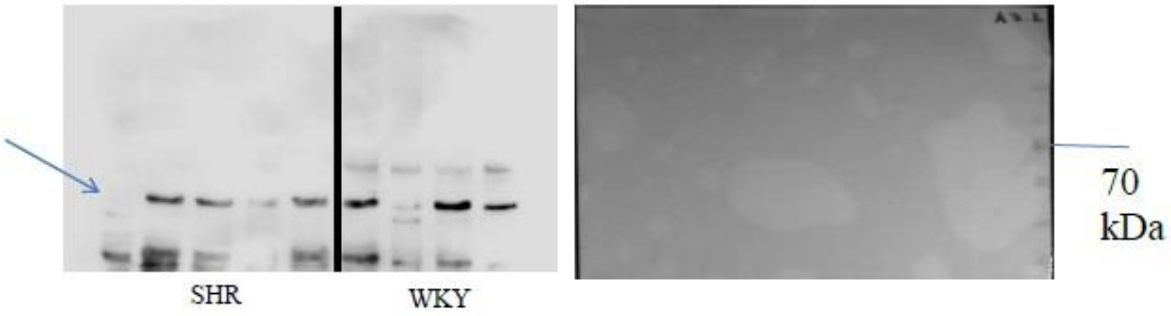
B) PKA, 4 strippings were performed before the blotting with PKA. PKA and β-actin were from the same membrane.



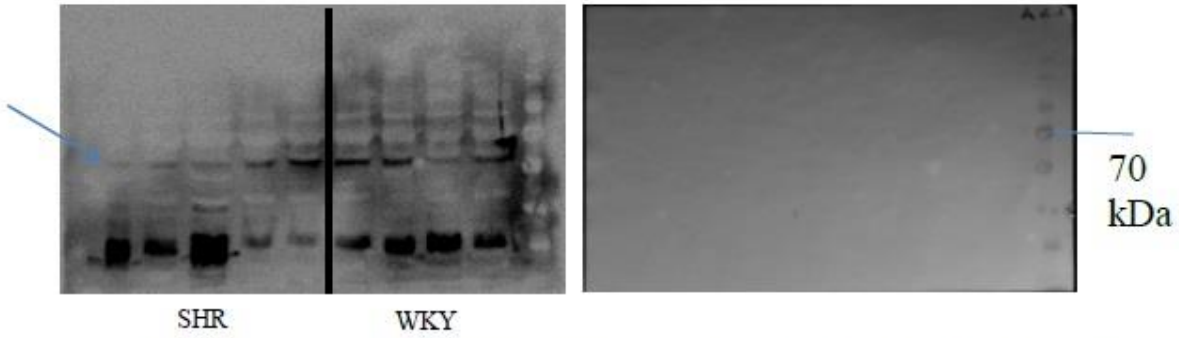
C) Perilipin, 2 strippings were performed before the blotting with perilipin. Perilipin and β-actin were from the same membrane.



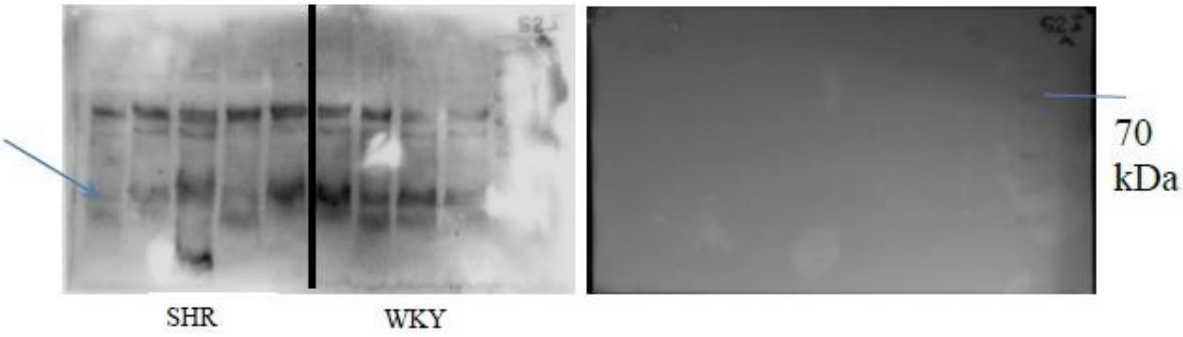
D) β-actin used to normalize β_1-AR, adenosine A1 receptor, GR, Gs, CGI-58 and HSL.2 strippings were performed before the blotting with β-actin.



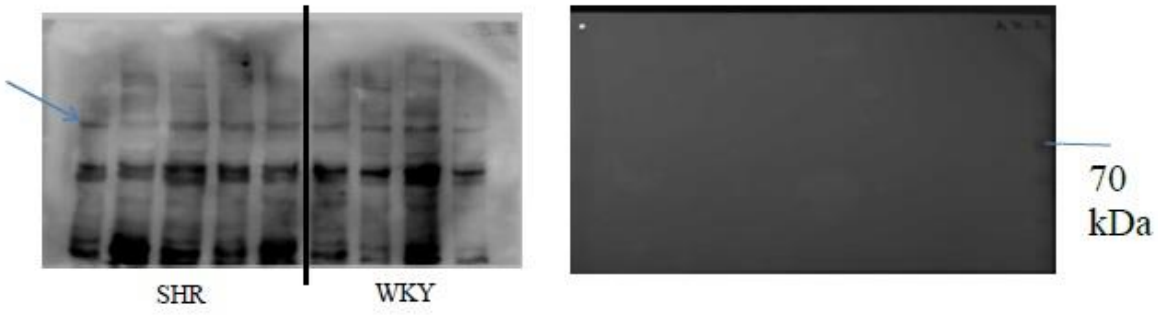
E) β_1-AR, 2 strippings were performed before the blotting with β_1-AR. β_1-AR and β-actin were from different membranes containing the same samples.



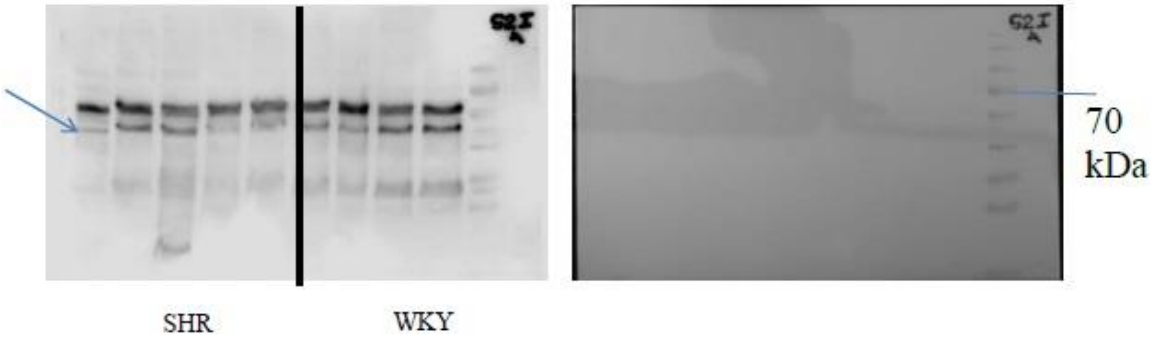
F) Adenosine A1 receptor, 2 strippings were performed before the blotting with adenosine A1 receptor. Adenosine A1 receptor and β-actin were from different membranes containing the same samples.



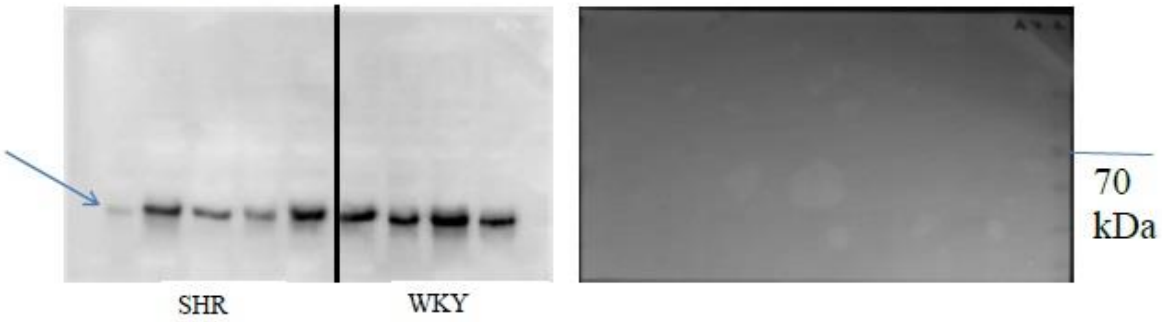
G) GR, 1 stripping was performed before the blotting with GR. GR and β-actin were from the same membrane.



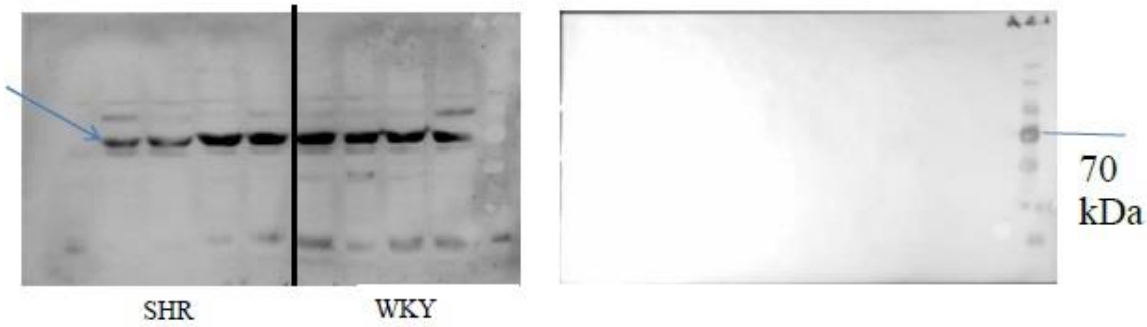
H) Gs, no stripping was performed before the blotting with Gs. Gs and β-actin were from different membranes containing the same samples.



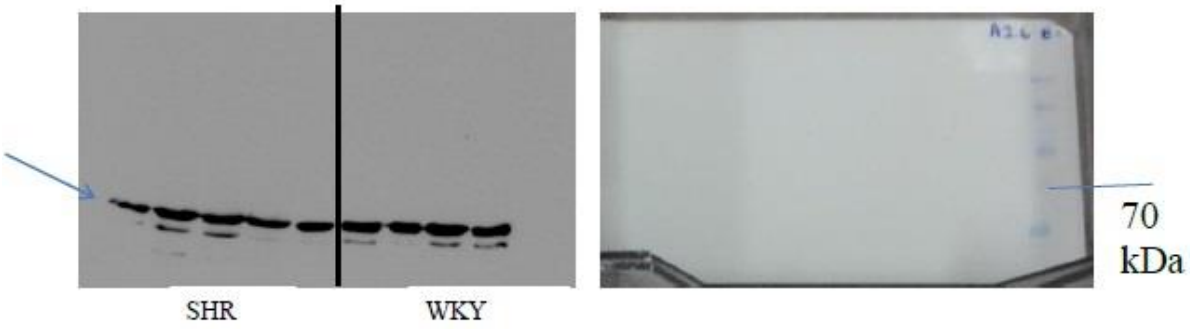
I) CGI-58, 4 strippings were performed before the blotting with CGI-58. CGI-58 and β-actin were from the same membrane.



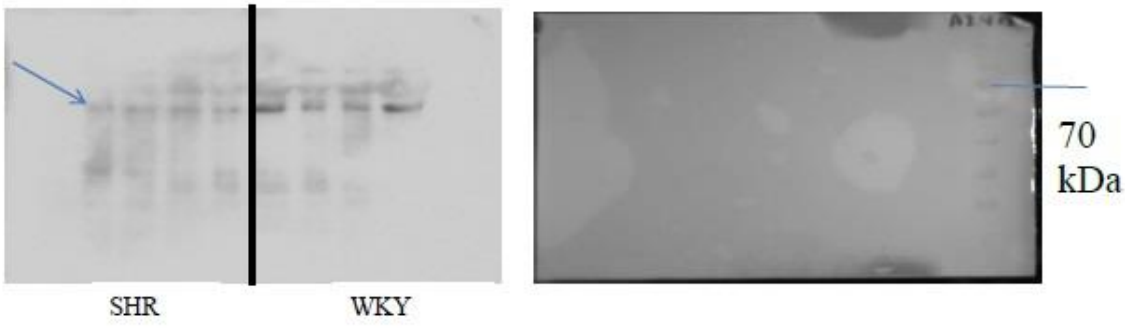
J) HSL, 1 stripping was performed before the blotting with HSL. HSL and β-actin were from the same membrane.



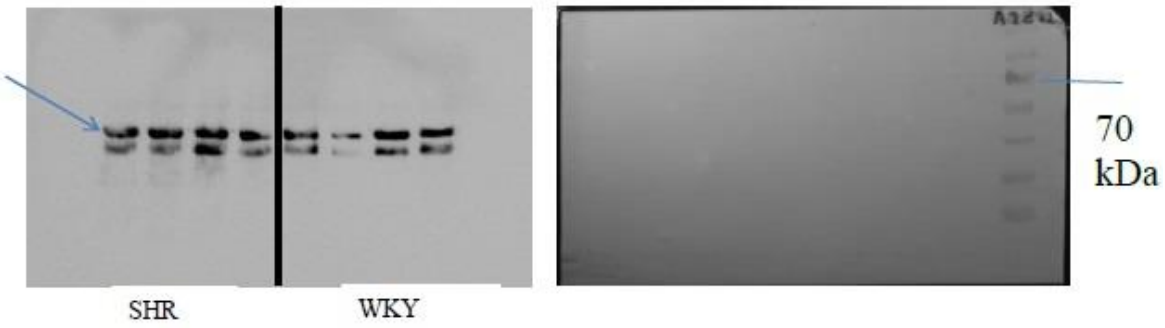
K) β-actin used to normalize adenosine A2 receptor and Gi. No stripping was performed before the blotting with β-actin



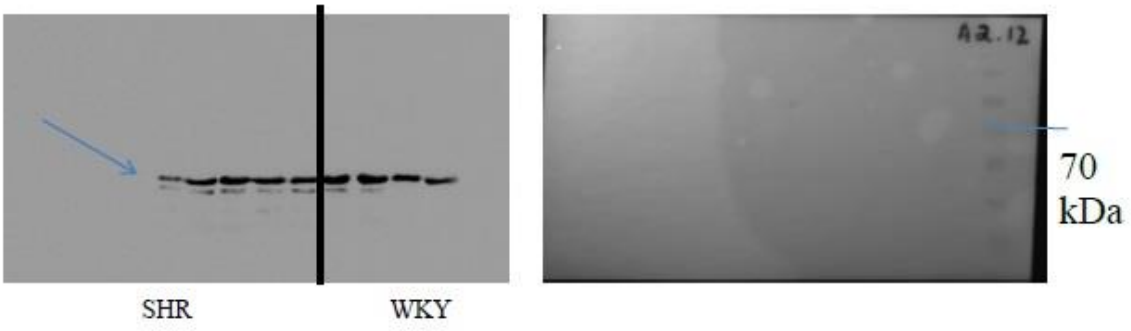
L) Adenosine A2 recptor, 5 strippings were performed before the blotting with adenosine A2 recptor. Adenosine A2 recptor and β-actin were from different membranes containing the same samples.



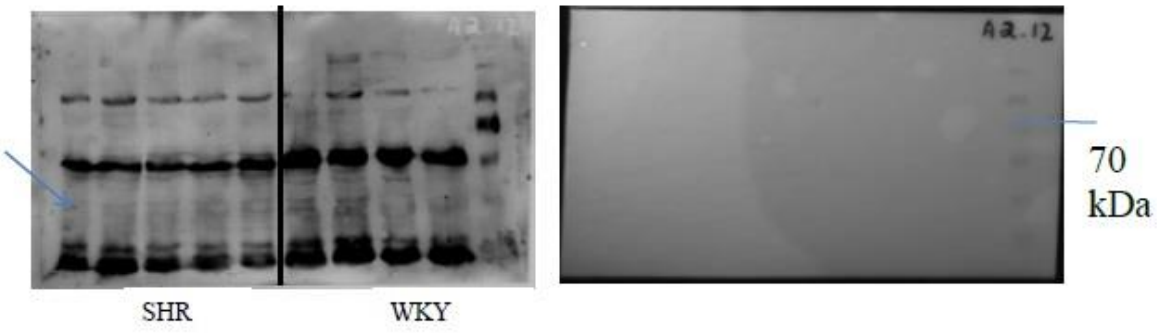
M) Gi, 1 stripping was performed before the blotting with Gi. Gi and β-actin were from different membranes containing the same samples.



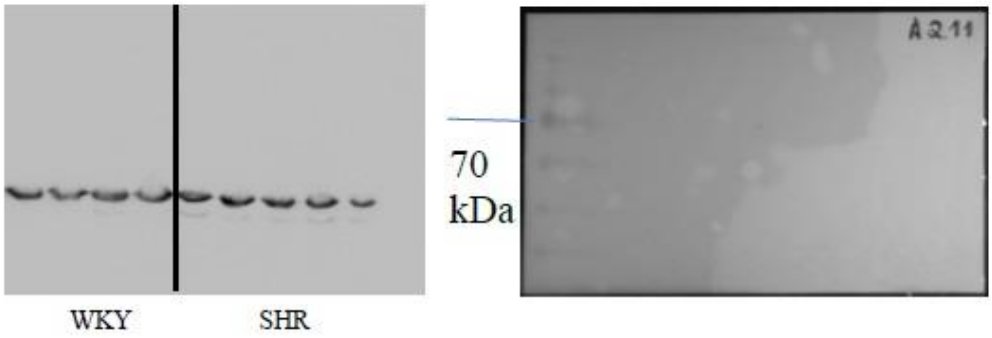
N) β-actin used to normalize β_3-AR.1 stripping was performed before the blotting with β-actin.



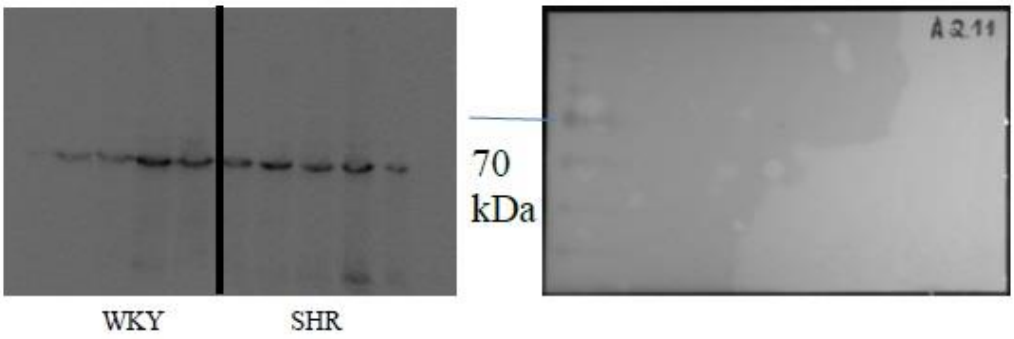
O) β_3-AR, no stripping was performed before the blotting with β_3-AR. β_3-AR and β-actin were from the same membrane.



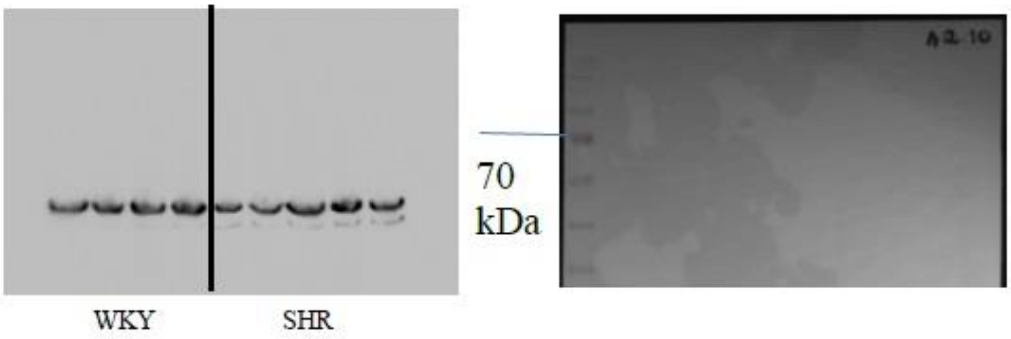
P) β-actin used to normalize β_2-AR.1 stripping was performed before the blotting with β-actin.



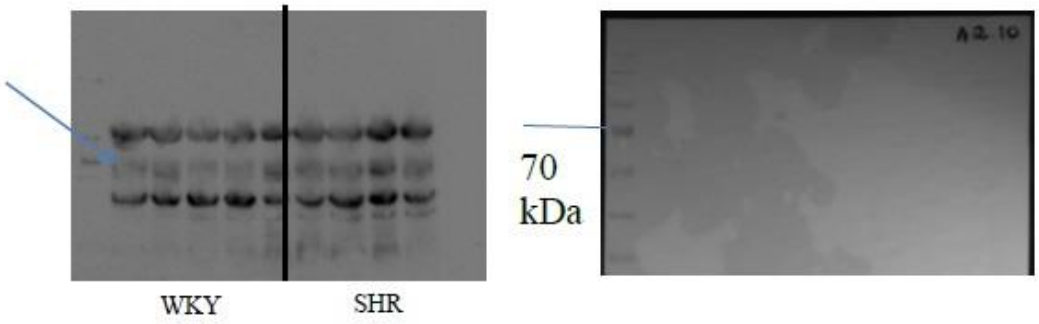
Q) β_2-AR, no stripping was performed before the blotting with β_2-AR. β_2-AR and β-actin were from the same membrane.



R) β-actin used to normalize ATGL.1 stripping was performed before the blotting with β-actin.



S) ATGL, 2 strippings were performed before the blotting with ATGL. ATGL and β-actin were from the same membrane.



Discussão

O estudo longitudinal de ratos de diferentes linhagens, isogênicas ou não, nos remete a conhecimentos da fisiologia básica descritos no final do século XIX pelo pesquisador Rubner (Rubner, 1883). Desta forma primeiramente, devemos considerar que os diferentes tamanhos dos animais, determinados pela relação superfície-peso influenciam a evolução de parâmetros que variam de modo alométrico ou seja, exponencial e não linear com o peso corpóreo. Estes parâmetros que então sofrem influência da alometria são: o consumo de alimento e a taxa metabólica de repouso, pois segundo este conceito, quanto menor o peso do animal, maior a sua relação superfície-peso, e maior a energia dispendida para se manter a temperatura corpórea e consequentemente o consumo alimentar (Tschöp *et al.*, 2012).

Este fenômeno foi coerente com os resultados de evolução de peso, consumo alimentar e taxa metabólica de respouso dos nossos animais dentre a 7ª e a 14ª semana de vida. Assim, ambos os grupos do modelo de hipertensão induzida (controle Wistar, grupo W e o hipertenso induzido por L-NAME, grupo L-NAME) apresentaram o mesmo ganho de peso, o que reduziu a relação superfície-peso na mesma proporção, e desta forma diminui a taxa metabólica para manter a temperatura corpórea e o consumo alimentar da mesma maneira. Diferentemente, no modelo de hipertensão genética, o grupo SHR apresentou maior ganho de peso em relação ao seu controle WKY, embora o seu peso fosse inferior do começo ao fim dos experimentos. Desta maneira, o SHR reduziu mais a sua relação superfície-peso, o que diminuiu de modo mais evidente a sua taxa metabólica de repouso e consequentemente o seu consumo alimentar, devido à redução da demanda energética para manter a sua temperatura. De modo semelhante, o grupo WKY, embora com peso sempre superior ao W, ganhou menos peso, o que diminuiu menos o seu consumo alimentar, porém não apresentou menor redução da taxa metabólica. Isto pode ser explicado pelo fato de que no final, a sua taxa metabólica de repouso foi inferior ao do Wistar. E finalmente, ambos os grupos hipertensos apresentaram o mesmo ganho de peso, que no entanto não resultou na mesma redução da ingestão alimentar e taxa metabólica entre eles, o que seria esperado, de acordo com a perspectiva alométrica. Ao contrário, o SHR apresentou menor redução no consumo alimentar se comparado com L-NAME, possivelmente devido so seu maior consumo alimentar que o grupo induzido no final. Importante destacar que apesar do menor ganho de peso do WKY em relação ao W ou do maior ganho de peso e maior redução da taxa metabólica e consumo alimentar do SHR em relação ao WKY, o grupo WKY exibiu os valores de peso corpóreo mais elevados que os outros grupos, da mesma forma que o SHR apresentou menor peso corpóreo e maior ingestão

alimentar e taxa metabólica de repouso do que os demais, em ambas as idades avaliadas (7 e 14 semanas de vida). Assim podemos concluir que o fator alométrico sofre influencia da linhagem estudada corrobrando com Gordon *et al.* (2016) (Gordon, Phillips e Johnstone, 2016). Esta e as demais diferenças encontradas na 14ª semana de vida foram relacionadas abaixo com os parâmetros séricos e moleculares de cada grupo.

Os nossos resultados séricos constataram a presença de um ambiente hormonal do estresse em ambos os grupos hipertensos (grupo L-NAME e SHR) e no grupo controle normotenso do modelo de hipertensão genética (grupo WKY). Este ambiente hormonal do estresse foi caracterizado por altas concentrações de catecolaminas e de corticosterona nos 3 grupos o que reflete, respectivamente, a ativação do SNS e do eixo HPA (Ishibashi *et al.*, 2013). Além disso, os ratos WKY apresentaram também altas concentrações do hormônio ACTH, como encontrado por outros estudos (Solberg *et al.*, 2001).

Os hormônios do estresse podem estar envolvidos com as alterações metabólicas exibidas pelo grupo hipertenso L-NAME, em comparação com seu controle W. A ativação do eixo HPA, é iniciada pela liberação hipotalâmica do hormônio CRH, o qual estimula os neurônios anorexigênicos do núcleo arqueado do hipotálamo (ARC), produtores de pró-opiomelanocortina (POMC), que dá origem ao hormônio α-melanócito estimulante (α-MSH) com efeito na diminuição da ingesta alimentar (Crespo *et al.*, 2014). Trata-se de uma resposta hipofágica ao estresse, observada em ratos sob estresse agudo (Maniscalco *et al.*, 2015). Embora não tenhamos medido as concentrações séricas de CRH, ele pode ser o hormônio atuante neste fenômeno, devido à ativação do eixo HPA constatada pelas altas concentrações de corticosterona.

A menor adiposidade do grupo L-NAME, observada pelo menor peso do panículo retroperitoneal e do tamanho dos adipócitos isolados deste e do depósito mesentérico, pode ser atribuída à menor ingestão alimentar e também ao efeito lipolítico dos hormônios do estresse, catecolaminas (Thorp e Schlaich, 2015) e corticosterona (Wang *et al.*, 2012). A lipólise mediada por estes hormônios é mais evidente no estresse agudo; pois cronicamente, as catecolaminas podem desencadear a dessensibilização de receptores β-adrenérgicos (Julius, Valentini e Palatini, 2000; Lambert *et al.*, 2010; Boer-Martins *et al.*, 2011), enquanto que a corticosterona pode induzir a lipogênese no tecido adiposo visceral (Fardet e Fève, 2014). Portanto, o menor consumo alimentar e menor adiposidade são duas respostas metabólicas típicas do estresse agudo, porém presentes no grupo L-NAME.

O tratamento com o L-NAME provocou o aumento da ingestão de água. Embora não tenhamos medido a angiotensina II e a aldosterona circulantes, a ativação do sistema renina-

angiotensina-aldosterona (SRAA) é bem descrita no modelo de hipertensão induzida por L-NAME (Takemoto *et al.*, 1997; Ikeda *et al.*, 2009; Suehiro *et al.*, 2015) e também pode ser atribuída aos hormônios do estresse, pois a exposição prolongada a altas concentrações de catecolaminas estimula o SRAA (Kishi e Hirooka, 2013). Desta forma, a angiotensina II, por estar elevada neste quadro, pode ter sido a responsável pelo estímulo dos centros da sede no hipotálamo (Bezalel *et al.*, 2015) e a aldosterona pode ter aumentado a reabsorção de sódio (Rossier, Baker e Studer, 2015), o que pode ter contribuído para a sede.

Com relação à lipólise dos adipócitos epididimais isolados, o grupo L-NAME não diferiu do seu grupo controle W, o que corrobora os valores de expressão das proteínas ATGL e CGI-58 que também foram os mesmos em ambos. Entretanto, a cafeína aumentou a liberação de glicerol basal apenas no grupo L-NAME, mas não em W. Neste caso, o efeito predominante da cafeína foi o de antagonizar os receptores de adenosina A1, retirando o seu efeito inibitório sobre a enzima adenilato ciclase, estimulando deste forma a lipólise mesmo na ausência de ligantes estimulatórios (Honnor, Dhillon e Londos, 1985). Possivelmente, este fenômeno foi evidente no grupo L-NAME devido à baixa expressão de duas proteínas: perilipinas, as quais formam uma barreira contra lipases em condições basais, o que aumenta a lipólise basal quando pouco expressa (Sztalryd e Kimmel, 2014); e receptores de adenosina A2, que são acoplados à proteína G estimulatória (Gs) (Panchal *et al.*, 2012), cujo antagonismo pela cafeína deve ter reduzido a lipólise de modo menos intenso no grupo L-NAME.

Embora não haja diferenças nos valores de resposta máxima ao isoproterenol (agonista não seletivo de β-adrenoceptores), nos grupos W e L-NAME, a expressão de algumas proteínas relacionadas à lipólise estimulada estava alterada no grupo hipertenso: alta expressão da HSL e baixa expressão de perilipinas e de receptores de adenosina A2, que aumentaria (Large *et al.*, 1998) e diminuiria (Panchal *et al.*, 2012; Sztalryd e Kimmel, 2014) a lipólise estimulada, respectivamente. Por se tratarem de efeitos antagônicos, nós sugerimos um processo de compensação, que não deve alterar a lipólise estimulada pelo agonista não seletivo de β-adrenoceptores no grupo L-NAME. Importante destacar também que a menor expressão de adrenoceptores β_1 no L-NAME, também não alterou a resposta máxima ao isoproterenol, que pode ser justificada pelo aumento compensatório da função de outros adrenoceptores, como o adrenoceptor β_2, como vamos discutir mais adiante.

O aumento da resposta máxima ao isoproterenol, na presença do antagonista do adrenoceptor β_2 ICI 118,551, sugeriu que este receptor associa-se mais a proteína Gi, do que a clássica Gs em ambos os grupos. Este resultado difere de anteriores (Farias-Silva *et al.*, 1999;

Grassi-Kassisse *et al.*, 2003), nos quais este antagonista não alterou a resposta lipolítica de ratos Wistar controle. Esta aparente contradição pode ser explicada pelos diferentes tipos de isoproterenol utilizados: hidro-cloridrato de (−)-isoproterenol do presente estudo e hidro-cloridrato de (±) isoproterenol de trabalhos prévios. O primeiro induz à fosforilação do adrenoceptor β_2 pela enzima PKA de modo mais intenso que o segundo (Sibley *et al.*, 1985), o que deve ter reduzido sua afinidade por Gs e aumentado a sua ligação por Gi (Xiao, 2001; Zamah *et al.*, 2002; Hill e Baker, 2003). Portanto, o antagonismo por ICI 118,551 sobre o adrenoceptor β_2 o libera da inibição exercida pela proteína Gi.

Os adipócitos do grupo L-NAME apresentaram aumento da lipólise mediada pelo adrenoceptor β_2, o que pôde ser observado pela maior resposta máxima ao salbutamol (agonista seletivo ao adrenoceptor-β_2) no L-NAME em comparação com W. Entretanto, a expressão de adrenoceptores β_2, Gs e Gi foi a mesma entre ambos os grupos; portanto outros mecanismos moleculares além da expressão protéica podem explicar este aumento de função do receptor, como aumento da sua afinidade aos hormônios e alterações na via de sinalização, como maior acoplamento e/ou função da proteína Gs, investigações não realizadas neste presente estudo.

A cafeína potencializou a lipólise estimulada por salbutamol apenas em W, mas não no L-NAME, diferentemente da lipólise basal, possivelmente devido ao efeito lipolítico mais intenso mediado por adrenoceptores-β_2 neste grupo, o qual gera grandes quantidades de AMPc. Deste modo, um incremento na disponibilidade de AMPc gerado pela inibição da fosfodiesterase e pelo antagonismo de receptores de adenosina A1 pela cafeína (Panchal *et al.*, 2012) deve ter pouco efeito frente à grande quantidade de AMPc no hipertenso, porém grande impacto no grupo controle.

O aumento da função de adrenoceptores β_2 corrobora estudos anteriores com modelos de estresse agudo em ratos (por choque nas patas e por natação), nos quais os animais apresentaram supersensibilidade ao isoproterenol, adrenalina e TA 2005 (potente agonista seletivo de adrenoceptores β_2) em adipócitos epididimais (Farias-Silva *et al.*, 1999) e no tecido cardíaco (Marcondes *et al.*, 1996; Vanderlei *et al.*, 1996), o que foi abolido por ICI 118,551. Entretanto, não houve aumento da sua expressão, diferentemente do que foi encontrado no modelo de estresse agudo em ratos por choques nas patas (Farias-Silva *et al.*, 2004). Este estudo constatou também uma diminuição da expressão de adrenoceptores β_1, β_3 e de GR em adipócitos epididimais isolados (Farias-Silva *et al.*, 2004); somente a redução de adrenoceptores β_1 corroborou nossos resultados, porém a expressão dos outros marcadores permaneceu a mesma entre W e L-NAME.

Os glicocorticoides são os responsáveis por estas alterações na expressão destes receptores (Bakopanos e Silva, 2002). Embora altas concentrações circulantes de corticosterona fossem observadas no grupo L-NAME se comparado ao seu controle, somente a expressão de adrenoceptores β_1 encontrou-se alterada. A heterogeneidade entre estes estudos pode ser explicada pelo estado hipertensivo, presente apenas neste estudo, e também pelo tipo e duração de diferentes agentes estressores, que neste caso é a hipertensão.

Podemos sugerir que o aumento da função de adrenoceptores β_2 pode aumentar a importância da adrenalina como importante estimulador endógeno da lipólise (Grassi-Kassisse *et al.*, 2003), o que pode contribuir para os distúrbios metabólicos associados à hipertensão. No presente estudo, este aumento de função dos adrenoceptores β_2 pode estar relacionado ao aumento da lipólise, observado na presença do agonista de adrenoceptores β_2 e da cafeína, associado com a reduzida adiposidade do grupo hipertenso.

Interessantemente, o grupo WKY também exibiu algumas alterações metabólicas observadas no grupo L-NAME quando comparados com o grupo W, o que pode ser atribuído aos hormônios do estresse, catecolaminas, corticosterona e ACTH, embora ele não desenvolva a hipertensão. As altas concentrações de ACTH no WKY, mas não no L-NAME, devem ser decorrentes de um prejuízo na função do seu eixo HPA, na qual a menor sensibilidade da pituitária aos glicocorticoides deve diminuir o seu efeito inibitório sobre esta glândula no mecanismo de retroalimentação negativa. Portanto deve haver uma menor inibição dos glicocorticoides sobre a produção de ACTH no WKY (Solberg *et al.*, 2001).

Uma destas alterações também encontradas no L-NAME, foi a menor ingestão alimentar do WKY na 7ª semana de vida. Trata-se portanto, da mesma resposta hipofágica ao estresse, exibido pelo L-NAME na 14ª semana de vida (ou seja, após a indução da hipertensão), que é desencadeado pelo hormônio CRH sobre neurônios produtores de POMC (Crespo *et al.*, 2014). Porém, diferentemente do que acontece no grupo L-NAME, o WKY aumenta o seu consumo alimentar, de modo a se igualar ao W; este aumento na ingestão é consistente com o efeito dos glicocorticoides pois inibem a produção de CRH e estimulam os neurônios produtores de neuropeptídeo Y (NPY) no ARC, o que aumenta o apetite e diminui o gasto energético (Crespo *et al.*, 2014). Trata-se portanto, de uma resposta hiperfágica ao estresse crônico, que sucede a hipofágica, desencadeada por exposição prolongada aos glicocorticoides (Yau e Potenza, 2013).

A menor taxa metabólica de repouso no WKY foi totalmente condizente com este efeito dos glicocorticoides sobre o NPY e também corroborou a menor expressão da proteína

desacopladora 3 no músculo esquelético. Isto porque ela promove o desacoplamento da fosforilação oxidativa mitocondrial na musculatura esquelética, o que dissipa calor e aumenta o gasto energético (Depieri *et al.*, 2004). Estudos com roedores sugerem que a exposição prolongada a catecolaminas (Depieri *et al.*, 2004) e a ação central crônica dos glicocorticoides (Zakrzewska *et al.*, 1999) pode reduzir sensivelmente a expressão de UCP3. Portanto, as alterações metabólicas exibidas pelo WKY no seu consumo alimentar e na sua taxa metabólica na 14ª semana devem ser decorrentes do efeito prolongado dos hormônios do estresse, se comparado com o L-NAME.

Apesar destas diferenças, o WKY exibiu também uma menor adiposidade, que pode ser atribuída aos efeitos lipolíticos das catecolaminas (Thorp e Schlaich, 2015), da corticosterona (Wang *et al.*, 2012) e do ACTH (Chaves, Frasson e Kawashita, 2011), além de maior ingestão hídrica, se comparado ao W, possivelmente desencadeada por altas concentrações de angiotensina II (Kishi e Hirooka, 2013) e de aldosterona (Rossier, Baker e Studer, 2015), da mesma forma que no grupo L-NAME.

Com relação ao modelo genético de hipertensão, SHR, este não diferiu de seu controle normotenso WKY nas concentrações séricas dos hormônios do estresse, catecolaminas e corticosterona; no entanto os valores séricos de ACTH foram menores quando comparados com os valores do WKY. E neste caso, estudos sugerem alterações no eixo HPA de ambos os grupos: a já mencionada menor sensibilidade da pituitária do WKY aos glicocorticoides, os quais apresentam menor capacidade de inibição da sua produção de ACTH (Solberg *et al.*, 2001), e também a menor produção deste hormônio no SHR (Gómez, De Kloet e Armario, 1998). Portanto, as diferenças metabólicas entre estes grupos encontradas neste estudo não podem ser atribuídas somente aos hormônios do estresse, pois com a exceção do ACTH, eles não diferem entre si. Deste modo, outra anormalidade endócrina pode explicar estas alterações entre os grupos: a hiperfunção da glândula tireoide no SHR (Wright *et al.*, 1978; Heckmann e Zimmer, 1992).

A hiperfunção desta glândula pode ser desencadeada pelos hormônios do estresse, por meio da estimulação noradrenérgica sobre o núcleo paraventricular do hipotálamo, o qual produz o hormônio liberador de tirotropina (TRH), que inicia a ativação do eixo hipotálamo-hipófise-tireoide (HPT) (Bruhn e Jackson, 1992). Os hormônios tireoideanos, por sua vez, estão envolvidos na modulação da pressão arterial e no desenvolvimento de hipertrofia cardíaca nos ratos SHR de modo que a tireoidectomia previne o desenvolvimento da hipertensão nesta linhagem (Heckmann e Zimmer, 1992). Em relação ao metabolismo energético, o hormônio tireoideano triiodotironina (T3) intensifica as funções metabólicas,

como a ingestão alimentar e hídrica, a termogênese, a taxa metabólica de repouso, a síntese, oxidação e secreção biliar de colesterol, a captação, oxidação e síntese de glicose e a captação de triacilgliceróis pelo fígado (Duntas e Brenta, 2012). Portanto, a identificação de valores elevados nos parâmetros metabólicos do SHR, como consumo de comida e taxa metabólica de repouso podem ser explicados por altas concentrações de T3. Este hormônio também aumenta a expressão e a função de UCP3 (Lombardi *et al.*, 2015). Neste estudo, este efeito deve ter se restringido ao aumento de função, uma vez que não foi observada uma diferença na expressão desta proteína. Frente às altas concentrações circulantes de corticosterona no SHR, nós hipotetizamos que a ação crônica e central deste hormônio na redução de UCP3 (Zakrzewska *et al.*, 1999) pode ter compensado o aumento por T3. Além disso, a reduzida adiposidade do grupo SHR pode ser resultado do aumento do seu gasto energético e também ao efeito lipolítico da tireotrofina (THS), hormônio produzido pela pituitária, cujas concentrações circulantes apresentaram-se elevadas em alguns estudos com o SHR (Bruhn e Jackson, 1992).

De modo semelhante, ambos os grupos hipertensos apresentaram o ambiente hormonal do estresse, no qual não diferiram nas concentrações séricas de corticosterona e ACTH. Entretanto, o grupo SHR apresentou menores concentrações de catecolaminas que o L-NAME, o que sugere que, nas nossas condições experimentais, a ingestão crônica de L-NAME pode ter causado uma maior ativação do SNS do que o desencadeado pela susceptibilidade genética do SHR. Apesar desta diferença pontual, as diferenças metabólicas entre ambos também foram atribuídas à hiperfunção da tireoide do SHR.

Neste contexto, é possível que altas concentrações circulantes do hormônio T3 tenham desencadeado a elevada ingestão alimentar e a alta taxa metabólica exibida pelo SHR em relação ao L-NAME, em ambos os momentos analisados (7 e 14 semanas). A menor adiposidade do SHR pôde ser atribuída ao seu elevado gasto energético, como também à possível elevação do hormônio TSH neste grupo, com efeito lipolítico, como já discutido, o que sugere que a anormalidade na tireoide possa ter se sobreposto ao efeito lipolítico exercido pelas altas concentrações circulantes de catecolaminas no grupo L-NAME.

Ambos os grupos de hipertensos também não diferiram no seu consumo hídrico ao final dos experimentos, o que sugere que ambas as hipertensões apresentem o mesmo grau de ativação do SRAA, nas nossas condições experimentais.

Em relação à resposta lipolítica de adipócitos isolados, a lipólise basal do grupo SHR não diferiu do WKY. No entanto, a expressão de duas proteínas relacionadas a este processo estava aumentada no SHR: a ATGL e a perilipina, o que deve aumentar e diminuir a lipólise basal, respectivamente (Bézaire e Langin, 2009; Sztalryd e Kimmel, 2014). Neste caso, nós

sugerimos um processo de compensação entre estes mecanismos antagônicos, o que explica a ausência de alterações na lipólise basal do grupo SHR.

A cafeína aumentou a liberação basal de glicerol, de modo semelhante, em ambos os grupos. Nesta situação, como já comentado, a cafeína age pelo antagonismo dos receptores de adenosina A1 e A2, liberando a enzima adenilato ciclase da inibição exercida pela adenosina via receptores A1, visto que estes predominam sobre o subtipo A2 (Rines, Verdeguer e Puigserver, 2015). Como o SHR não apresenta diferenças de expressão de ambos os receptores com relação ao WKY, o efeito da cafeína foi o mesmo em ambos, diferentemente do que ocorreu com o modelo de hipertensão induzida por L-NAME, no qual a ela deve ter aumentado a lipólise basal devido à sua menor expressão de receptores de adenosina A2.

Não houve diferenças na área sob a curva, na resposta mínima e na resposta máxima ao isoproterenol entre os grupos, o que corrobora com a ausência de alterações na expressão das proteínas da via da lipólise estimulada, como Gs, Gi, PKA e HSL e dos receptores de adenosina A1 e A2. Entretanto, o ensaio com antagonistas revelou diferenças importantes nas subpopulações de β-adrenoceptores.

O antagonista seletivo de adrenoceptor β_2, ICI 118, 551, aumentou a resposta máxima ao isoproterenol e sua área sob a curva no grupo SHR, enquanto estes parâmetros não foram alterados no grupo WKY. Este fenômeno sugere que o antagonismo sobre os adrenoceptores β_2 liberou um efeito inibitório destes receptores sobre a lipólise. Trata-se de um mecanismo já bem descrito pelos famosos experimentos do nobelista Lefkovitz (Zamah *et al.*, 2002), os quais demonstraram que o receptor adrenérgico β_2 pode assumir um papel promíscuo pois pode se associar a proteína Gi, em detrimento às clássicas proteínas Gs, quando a expressão do receptor encontra-se aumentada (Hill e Baker, 2003) ou sob condições extremas (Taylor, 1990), como por exemplo, no estresse (Liu *et al.*, 2009; Fu e Xiang, 2015).

Este mecanismo corrobora com os resultados da lipólise estimulada pelo agonista seletivo de adrenoceptor β_2, salbutamol, na presença e ausência de cafeína. O efeito da cafeína na lipólise mediada por este agonista se restringiu apenas à elevação na área sob a curva no grupo WKY. Entretanto, ela não só elevou este parâmetro no SHR, como também suas respostas mínima e máxima ao sabutamol. Deste modo, o efeito predominante da cafeína na lipólise estimulada foi a inibição da fosfodiesterase (Panchal *et al.*, 2012), o que aumenta a disponibilidade de AMPc gerado pela estimulação do adrenoceptor β_2, que foi mínimo no WKY devido à sua baixa participação no processo lipolítico em ratos sob condições normais (Lafontan, 2012). No entanto a ação da cafeína foi mais potente no SHR pois deve ter se

contraposto ao efeito inibitório da proteína Gi. Além disso, o aumento da expressão da perilipina no SHR não foi suficiente para aumentar a lipólise estimulada por agonista, provavelmente devido ao efeito inibitório desta associação do adrenoceptor-β_2 com Gi.

Esta associação com Gi não esteve relacionado à mudanças na expressão de proteínas, uma vez que a expressão de adrenoceptores β_2, Gs e Gi foi a mesma em ambos os grupos. Do mesmo modo, não houve diferenças na expressão de adrenoceptores $\beta_{1,}$ β_3 e GR. Estes resultados diferiram do modelo de hipertensão induzida por L-NAME e dos estudos prévios com modelo de estresse agudo por choque nas patas (Farias-Silva *et al.*, 2004), os quais constataram o aumento da lipólise mediada por adrenoceptores β_2.

Para se entender tais diferenças, o primeiro ponto a ser considerado é o ambiente hormonal do estresse ao qual os animais destes estudos e do atual trabalho estavam expostos. Tanto o WKY como o SHR apresentaram altas concentrações circulantes de glicocorticoides, que não diferiram entre si; por outro lado, somente o grupo hipertenso induzido por L-NAME e os ratos estressados por choque nas patas exibiram este perfil hormonal do estresse, mas não seus respectivos controles. Como os glicocorticoides são os principais mediadores do aumento de expressão de adrenoceptores β_2 e da diminuição dos adrenoceptores β_1, β_3 e GR (Bakopanos e Silva, 2002), as mesmas concentrações circulantes de corticosterona devem ter causado o mesmo efeito no WKY e no SHR.

Outro ponto a ser considerado, além dos glicocorticoides, é o estado hipertenso na linhagem SHR, que se manifesta espontaneamente a partir da 4ª a 6ª semana de vida (Zicha e Kunes, 1999; Clifford, Dampney e Carrive, 2015). Este maior tempo de hipertensão no SHR pode ter desencadeado esta associação dos adrenoceptores β_2 às proteínas Gi (Taylor, 1990), com função protetora, na qual se opõe à lipólise mediada pelos receptores β_1 e β_3, mecanismo já descrito no coração de roedores (Xiao, 2001; Liu *et al.*, 2009; Fu e Xiang, 2015). Este mecanismo protetor impede a superestimulação na contração cardíaca, por se contrapor aos efeitos estimulatórios mediados pelos adrenoceptores β_1 e β_3 e é densencadeado por altas concentrações de catecolaminas circulantes, na resposta ao estresse (Liu *et al.*, 2009; Fu e Xiang, 2015). Além disso, esta via também estimula a sobrevivência celular dos cardiomiócitos, via fosfatidilinositol 3-quinase (PI3K)-serina-treonina quinase (Akt), iniciada pela subunidade $\beta\gamma$ da proteína Gi (Xiao, 2001; Fu e Xiang, 2015). Se o acionamento desta via também ocorre pela proteína Gi em adipócitos é outra questão que merece ser desvendada.

Em resumo, os nossos resultados constataram algumas alterações metabólicas em ambos os grupos hipertensos e no normotenso WKY, que podem ser atribuídas aos hormônios do estresse.

Embora o grupo L-NAME tenha desenvolvido a hipertensão pela ingestão crônica de L-NAME, ele exibiu alterações metabólicas típicas do estresse agudo, como a redução da ingesta e da adiposidade além do aumento da lipólise mediada por adrenoceptores β_2. Estes resultados diferem de outros provavelmente devido ao menor tempo de indução da hipertensão se comparado com outros estudos (Cardoso *et al.*, 2013), ou talvez por causa do uso de linhagens mais susceptíveis a alterações metabólicas de outros trabalhos, como os ratos Sprague Dawley (Roy, Perreault e Marette, 1998; Shankar *et al.*, 1998; Higaki *et al.*, 2001).

Por outro lado, os ratos WKY exibiram algumas alterações do estresse crônico, talvez atribuídas ao seu maior tempo de exposição aos glicocorticoides (Will, Aird e Redei, 2003), se comparado ao grupo L-NAME.

Além do mais, os ratos do grupo SHR também apresentaram altas concentrações dos hormônios do estresse da mesma forma que seu controle WKY, e também o hipertenso L-NAME; por este motivo, as diferenças entre seus parâmetros metabólicos foram atribúidas à sua hiperfunção na tireoide, a qual também pode ser decorrentes dos efeitos dos hormônios do estresse. Os estudos sobre metabolismo energético do SHR são conflitantes. O presente trabalho corroborou um estudo (Oliveira *et al.*, 2009), mas não outros (Iritani *et al.*, 1977; Hulman, Falkner e Chen, 1991; Reaven e Chang, 1991; Hajri *et al.*, 2001; Potenza *et al.*, 2005; Potenza *et al.*, 2006), os quais identificaram a presença de distúrbios metabólicos claramente associados com a síndrome metabólica. Esta aparente contradição pode ser justificada por diferenças na idade dos animais, pois o desenvolvimento destes distúrbios ocorre com a maturação (Oliveira *et al.*, 2009) e também pela grande diversidade genética dentre as sublinhagens de ratos SHR, cujas alterações metabólicas dependem da sua susceptibilidade em manifestá-las (Zhang-James, Middleton and Faraone, 2013). Este mecanismo de associação dos adrenoceptores β_2 às proteínas Gi pode explicar alguns estudos na literatura, que relatam uma menor lipólise mediada por agonistas β-adrenérgicos em ratos SHR (Spitzer, Burns e O'malley, 1985; Nelson, Shepherd e Spitzer, 1987; Chiappe De Cingolani, 1988) e em humanos hipertensos (Townsend e Klein, 1997).

Conclusões

O presente estudo sugere a presença de um ambiente hormonal do estresse intrínseco ao estado hipertensivo, e que pode estar relacionado à gênese de distúrbios metabólicos na hipertensão. Ele também fornece fortes indícios de que a possível dessensibilização de β-adrenoceptores na hipertensão não ocorra pelo processo de internalização dos mesmos por estímulo crônico das catecolaminas como foi anteriormente sugerido (Julius, Valentini e Palatini, 2000; Thorp e Schlaich, 2015), mas sim pela ação dos glicocorticoides sobre subpopulações distintas de β-adrenoceptores somada à associação da subpopulação β_2 à proteína Gi, que neste estudo, ocorreu independentemente das concentrações de catecolaminas e de corticosterona. Neste caso, outro fator intrínseco ao estado hipertensivo deve desencadear tal associação.

Entretanto a verificação destes mecanismos em adipócitos de seres humanos hipertensos, o seu processo de regulação, assim como suas consequências fisiológicas são questões que permanecem para serem desvendadas.

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A**nexos**

Anexo 1: Documento de aprovação pelo Comitê de Ética em Experimentação Animal

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O,

Certificamos que o projeto **"ESTUDO DA ATIVIDADE LIPOLÍTICA EM ADIPÓCITOS ISOLADOS DE PANÍCULOS ADIPOSOS DE DIFERENTES MODELOS DE RATOS COM ATIVIDADE SIMPÁTICA ESTIMULADA TRATADOS OU NÃO COM MELATONINA"** (protocolo nº **2616-1**), sob a responsabilidade de **Profa. Dra. Dora Maria Grassi Kassisse / Larissa Yuri Ishizu**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **13 de fevereiro de 2012**.

Campinas, 13 de fevereiro de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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Anexo 2: Adendo ao documento de aprovação pelo Comite de Ética em Experimentação Animal

Campinas, 06 de novembro de 2015

Á Comissão de Ética no Uso de Animais (CEUA/UNICAMP)

Ref.: Solicitação de alteração de título de projeto e informação sobre mudança de metodologia utilizada (protocolo nº 2616-1).

Vimos por meio deste adendo, solicitar a alteração do título do projeto "Estudo da atividade lipolítica em adipócitos isolados de panículos adiposos de diferentes modelos de ratos com atividade simpática estimulada tratados ou não com melatonina" (protocolo nº 2616-1) para **"**Estudo da atividade lipolítica de adipócitos isolados do tecido adiposo epididimal e do metabolismo energético de ratos provenientes de dois modelos de hipertensão: genética e induzida**".**

Informamos também que os grupos tratados com dieta hiperlipídica e com melatonina, que teriam seus tecidos analisados, foram excluídos do projeto original. Os animais pertencentes a estes grupos foram utilizados para análise dos mesmos tecidos anteriormente descritos, através da técnica de *Western Blotting*, e também para a retirada de outros tecidos visando seu uso em ensaios futuros e complementares a esta investigação.
Declaramos ainda que não houve alteração em relação ao número de animais utilizados.

Justificativa:
Considerando que no campo da pesquisa metabólica ainda são necessários maiores estudos relacionados à interação adrenérgica na hipertensão e a associação desta aos distúrbios metabólicos, optamos por realizar uma investigação mais aprofundada do mecanismo de ação envolvido na interação dos receptores adrenérgicos relacionados ao desenvolvimento da hipertensão em modelo com atividade simpática exacerbada. Dessa forma, optamos por não realizar o estudo com dieta hiperlipídica, bem como o tratamento com melatonina, descritos no projeto inicial e adicionamos ao trabalho um amplo estudo molecular utilizando a técnica *Western Blotting*. Destes animais foram também retirados os seguintes órgãos: músculo gastrocnêmio, fígado, rim, pulmão, hipotálamo e adrenais para futuros estudos. Ressaltamos que não houve alteração no número de animais inicialmente proposto para este projeto.

Larissa Yuri Ishizu
Larissa Yuri Ishizu

Profª Drª Dora Maria Grassi Kassisse
ORIENTADORA
Departamento de Biologia Estrutural e Funcional
Instituto de Biologia-UNICAMP

Anexo 3: Declaração de que a dissertação ou tese não infringe os dispositivos da lei nº 9610/98, nem o direito autoral de qualquer editora

Profa. Dra. Rachel Meneguello
Presidente
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"Estudo da atividade lipolítica de adipócitos isolados do tecido adiposo epididimal e do metabolismo energético de ratos provenientes de dois modelos de hipertensão: genética e induzida"**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 17 de dezembro de 2015.

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